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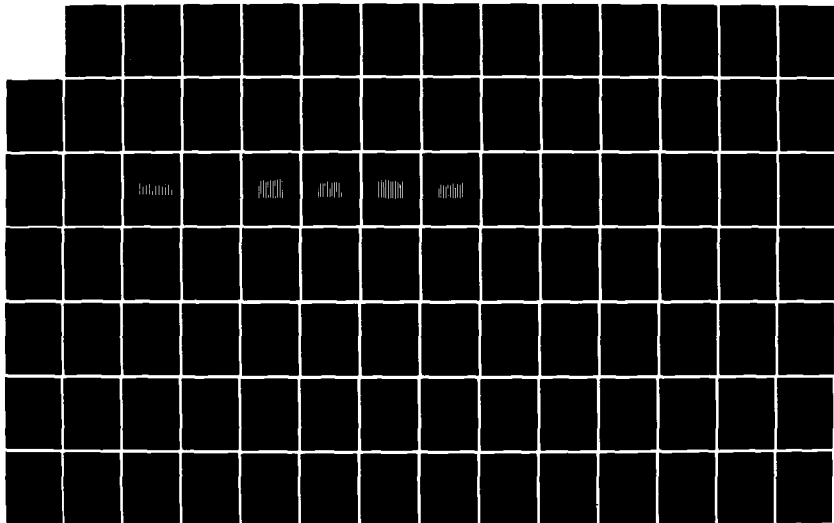
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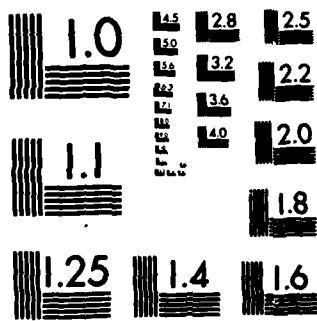
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REPORT #6

Host Defense Against Opportunist Microorganisms Following Trauma

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D
H. Stephen Bjornson, M.D., Ph.D.
William A. Altmeier, M.D.
Josef E. Fischer, M.D.

AUGUST 31, 1982

Supported by

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**The Christ Hospital Institute of
Medical Research
Cincinnati, Ohio 45219**

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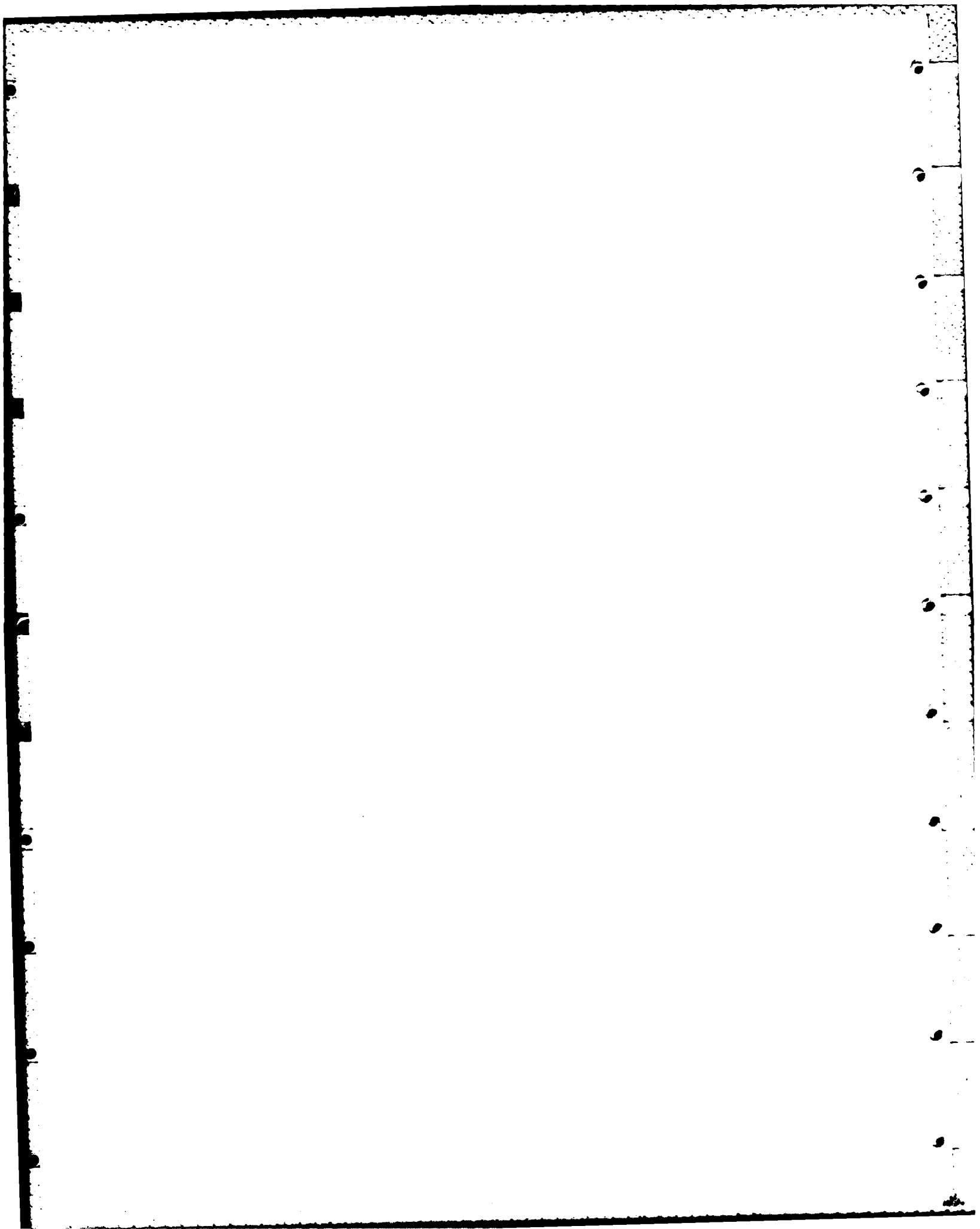
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→ as assessed by measurement of total caloric and protein intake. Burn serum inhibitor(s) of PMNL function were shown to be heat-stable and distinct from IgG and proteases with sensitivity to inactivation by benzamidine hydrochloride. Reduction in alternative pathway activity was not found to be caused by an aberrant factor that augments the functions of the regulatory proteins, H and I, or to elevation of these proteins as compared with C3 and B. Rather, this abnormality was associated with diminution in the functional activity of D. Preliminary evidence was provided to suggest that the burn serum inhibitor(s) of alternative pathway activity and PMNL function are alpha globulin-associated and bear a relationship with burn serum inhibitor(s) of lymphocyte function.



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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, 1978.

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We express our appreciation to Dr. Susan R. Watson, Department of Medicine, University of Cincinnati College of Medicine, for many helpful discussions regarding the lymphocyte data, to Dr. John D. Crissman, formerly of the Department of Pathology, University of Cincinnati College of Medicine, for performing the histological studies described in this report, and to Dr. Clark D. West, Cincinnati Children's Hospital Research Foundation, for the gift of antiserum to human H.

SUMMARY

Previous studies from our laboratory have demonstrated reduction in alternative complement pathway activity and serum-mediated inhibition of bacterial phagocytosis by polymorphonuclear leukocytes (PMNL) following burn injury in humans. In the present investigation, the abnormalities were documented in a guinea pig model of burn injury, and preliminary evidence was presented to suggest that burn wound infection is a primary determinant in induction of these abnormalities. Studies in burned humans failed to demonstrate a correlation between the abnormalities and nutritional status as assessed by measurement of total caloric and protein intake. Burn serum inhibitor(s) of PMNL function were shown to be heat-stable and distinct from IgG and proteases with sensitivity to inactivation by benzamidine hydrochloride. Reduction in alternative pathway activity was not found to be caused by an aberrant factor that augments the functions of the regulatory proteins, H and I, or to elevation of these proteins as compared with C3 and B. Rather, this abnormality was associated with diminution in the functional activity of D. Preliminary evidence was provided to suggest that the burn serum inhibitor(s) of alternative pathway activity and PMNL function are alpha globulin-associated and bear a relationship with burn serum inhibitor(s) of lymphocyte function.

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I. ORIGINAL OBJECTIVES

- A. To determine the effects of burn injury on classical and alternative complement pathway activity and on serum phagocytosis-promoting activity using a burned animal model.
- B. To correlate changes in the humoral parameters with burn size, weight loss, burn wound colonization, and bacteremia.
- C. To further investigate the occurrence and duration of serum-mediated inhibition of phagocytosis by polymorphonuclear leukocytes (PMNL) in human burned patients.
- D. To determine the relationship between this abnormality and burn size, age, number and type of infectious complications, and nutritional status.
- E. To fractionate the inhibitors of PMNL phagocytosis and alternative complement pathway activity from human burn sera.
- F. To determine the relationship of the two burn serum inhibitors to each other and to the burn serum inhibitor of T-lymphocyte blastogenesis.

II. BACKGROUND

The complement system is the primary humoral mediator of biological events associated with host resistance against microbial infection including changes in vascular permeability, chemotaxis of PMNL and monocytes, opsonization leading to phagocytosis of bacteria and yeasts, bactericidal and viricidal activity, and neutralization of viruses. In recent years, considerable information has been reported regarding alterations of complement associated with burn injury. However, the causes of these alterations and their relationship to abnormalities of cellular functions associated with burn injury are poorly understood.

Diminution in total hemolytic complement and concentrations of C1, C4, C2, and C3 during the first week postburn was originally documented in burned humans by Fjellstrom and Arturson (1). Subsequent studies by other investigators confirmed this observation in burned humans (2-6), rats (7), and dogs (8, 9). In one of these studies (7), additional complement components (C5, C8, and C9) were measured, and reduction in C5 and C8 was demonstrated. It has been postulated that mechanical leakage of complement from the burn wound (5) or consumption of complement by heat-altered tissue (10) may be responsible for the observed abnormalities.

The temporal sequence of alterations of complement in burned humans and the relationship of the alterations to the severity and type of burn injury and to the occurrence of infectious complications have been investigated in our laboratory. Reduction in total hemolytic complement (11-14) and immunochemical concentrations of C1, C4, C2, C3, and C5 (12-14) was demonstrated during the first 10 days postburn in the sera of selected patients with large full-thickness injuries and in a majority of patients who subsequently developed septic episodes. Alternative pathway-mediated conversion of C3 by inulin (11-16) and cobra venom factor (CoVF) (13, 14, 16) and the immunochemical concentration of P¹ (properdin) (11-14) were found to be decreased for up to 50 days postburn in patients with 20% or greater total body surface burns. The most marked reduction in alternative pathway-mediated C3 conversion was demonstrated in patients with large full-thickness injuries and infectious complications (16). Elevated concentrations of B, C3, and I, a regulatory protein of the complement system, were observed during 50 days postburn; the most pronounced increases in these proteins were observed in patients with small burns (11-14). No difference in the occurrence of the complement abnormalities was noted in patients with burns caused by flame, immersion scald, or acid contact (11).

Consumption of complement was documented during septic episodes (11-14). Multiple patterns were observed as follows: a) consumption of C1-C5, b) consumption of C1 and C4, c) consumption of C1, C4, and C2, d) consumption of C1, C4, C2, and C5, but not C3, and e) consumption of C1-C5, B, and I (12,13). This latter pattern was observed primarily in patients who died of septic shock (12,13).

¹ Nomenclature of the alternative complement pathway recommended by the World Health Organization (WHO Bulletin 59:489-491, 1981) has been used in this report.

Reduction in the immunochemical concentration of C3 during septic episodes has been confirmed by other investigators (17, 18). In addition, patients with septic episodes during their clinical course have been shown to have lower levels of C3 initially following burn injury than other patients (17, 18). Persistent diminution in the immunochemical concentration of P in burned patients has also been confirmed in several studies (17-22).

Studies to determine the cause of the reduction in alternative pathway-mediated C3 conversion associated with burn injury have implicated a serum inhibitor. Supplementation of burn sera with normal human serum was not shown to correct the abnormality (11, 15). Dialysis of burn sera or incubation of burn sera with dialysis tubing markedly increased C3 conversion without causing complement consumption, suggesting that an inhibitory activity had been removed or inactivated by dialysis and/or interaction with cellulose (16).

The effects of burn injury on the serum factors which facilitate bacterial opsonization have been investigated by us (3, 11-14) and by Alexander et al. (17-21). In most of these studies, the ability of patients' sera to support phagocytosis and killing by normal human PMNL of laboratory adapted bacterial test strains was measured (3, 11, 17-21). Reduction in opsonic activity was demonstrated in patients' sera during the first 2 weeks postburn and was restored to normal thereafter. In several of these studies (11, 18, 19), decreased opsonic activity was also demonstrated during septic episodes and was associated with complement consumption. In other studies in which opsonic activity of septic burned patients' sera for the specific infecting bacterial strains was measured, complement consumption was generally not associated with reduction in opsonic activity, and decreased opsonic activity immediately following burn injury was generally not demonstrated (12-14).

The observation that multiple complement abnormalities did not decrease the opsonic activity of burned patients' sera for the bacteria causing septic complications led us to postulate that immune antibacterial IgG antibodies might be produced during burn wound colonization which could facilitate opsonization of the bacteria in the absence of an intact complement system. However, when this hypothesis was tested experimentally, no increase in heat-stable opsonic activity or agglutinating antibodies directed against the infecting bacterial strains was demonstrated in the burn sera (14). One explanation for these findings is that sufficient natural IgG antibodies were present in the burn sera to facilitate opsonization of the bacteria in the presence of minimal complement activity. In this regard, augmentation of alternative pathway-mediated opsonophagocytosis of bacteria by IgG antibodies has been recently reported by Edwards et al. (23). Alternatively, the methodology used for measurement of opsonic activity might not have been sensitive enough to detect minor defects. In this regard, Allen and Pruitt have recently demonstrated diminution in serum opsonic activity in burned patients associated with septic episodes by measuring zymosan-induced luminol-dependent chemiluminescence (24).

A yeast opsonic defect has also been reported to occur following burn injury (22). In addition, fibronectin has been shown to be decreased in burn sera (25,26). Although this protein participates in clearance of certain particulate matter by the reticuloendothelial system, its role in bacterial opsonization is ancillary (27).

In all of the studies cited above in which opsonic activity has been measured, the concentrations of serum tested in the assays ranged from one to ten percent. Recent preliminary results from our laboratory have shown that the sera from certain burned patients inhibit the bactericidal activity of normal human PMNL when tested at a physiological concentration (28). Inhibitory activity was demonstrated in the sera of three of 12 bacteremic burned patients during 13 to 56 days postburn. Decreased bactericidal activity was related to an inhibitory effect of the burn sera on the phagocytic process, which reduced the number of bacteria internalized and killed intracellularly. The inhibitory effect was shown to involve a direct interaction of the burn sera with the leukocytes, which was not associated with cell death and was not reversed by washing of the leukocytes. The inhibitory activity was detected in dialyzed sera, suggesting that it may be distinct from the inhibitory activity responsible for abnormal alternative pathway-mediated C3 conversion.

The sole use of burned humans as subjects for study in our investigation and those of others has precluded discovery of the causes and biological significance of abnormalities of humoral and cellular factors associated with burn injury. Numerous variables cannot be controlled, i.e. variation in total patient management, age, anatomical location of burn wound, burn size, pre-burn nutritional and clinical status, and administration of blood products, antibiotics, and other drugs. For this reason, the investigation described in this report will focus on the use of a burned animal model.

III. EXPERIMENTAL APPROACH

The effects of burn injury on various humoral parameters of host defense were to be investigated using a burned guinea pig model. The guinea pig was selected for study, because the complement system of this animal closely resembles that of the human (29-33). In addition, controlled studies have shown that the metabolic response of growing guinea pigs to severe burn injury simulates the human postburn metabolic response (34).

Three groups of growing Hartley guinea pigs containing 110 animals in each group were to be studied. The first two groups of animals were to receive scald burn injuries of 25% and 50% of the total body surface respectively. The third group of animals was to serve as the sham-burned controls. Histological examination was to be performed on selected animals in each burned group to insure that full-thickness injuries had been produced without damage to the visceral organs. On the day of burning or sham-burning and at 5-day intervals during 50 days postburn, ten animals from each group were to be anesthetized and weighed; blood for preparation of blood cultures and sera was to be collected prior to sacrifice. Quantitative burn wound cultures were also to be obtained from the burned animals.

Sera were to be tested for total hemolytic complement and alternative pathway-mediated complement consumption by inulin. The ability of the sera at concentrations of 10%, 50%, and 98% to facilitate phagocytosis of Staphylococcus aureus 502A and Escherichia coli 075 by casein-induced peritoneal PMNL from non-burned guinea pigs was also to be determined. The data from each group at the various time intervals were to be collated and analyzed by analysis of variance. Changes in the various humoral parameters were to be correlated with burn size, change in weight, burn wound colonization, and bacteremia.

Studies were to be continued in burned humans to further investigate abnormal alternative complement pathway activity and serum-mediated inhibition of bacterial phagocytosis by normal human PMNL. All patients admitted to the Cincinnati General Hospital during the contract period with burns involving greater than 20% of the total body surface but without associated medical problems were to be studied. Blood specimens for preparation of sera were to be obtained at weekly or biweekly intervals for 6 to 8 weeks postburn. Nutritional status and infectious complications in the patients were to be documented. Nutritional status was to be monitored by tabulation of daily total caloric and protein intake.

C3 conversion by inulin and CoVF was to be measured in the serial serum samples from each patient to screen for abnormal alternative complement pathway activity. The sera were also to be tested for their ability to inhibit phagocytosis by normal human PMNL of S. aureus 502A and E. coli 075 opsonized with pooled normal human serum. The occurrence and duration of serum-mediated inhibition of phagocytosis by PMNL was to be correlated with burn size, age, number and type of infectious complications, and nutritional status.

Sera from five burned patients with abnormal alternative complement pathway activity, two burned patients with abnormal alternative pathway activity and an inhibitory effect on bacterial phagocytosis by PMNL, and five healthy adult donors were to be fractionated by DEAE-cellulose chromatography. The conditions previously used for fractionation of immunosuppressive activity from human burn sera (35) were to be used. Protein peaks were to be desalted by chromatography on Sephadex G-25 and lyophilized. The peaks were to be solubilized and tested for their ability to inhibit phagocytosis by normal human PMNL of S. aureus 502A and E. coli 075 opsonized with pooled normal human serum, C3 conversion by inulin and CoVF in pooled normal human serum, and the proliferative response of normal human peripheral blood lymphocytes (PBL) to phytohemagglutinin (PHA).

IV. PROGRESS REPORT

A. Animal Studies

1. Results

a. Development of a guinea pig model of burn injury

The conditions described by Herndon et al. (36) were used in our initial experiments. Twenty male and female Hartley guinea pigs weighing approximately 300 g were purchased from Murphy Breeding Labs, Inc., Plainfield, IN. Following delivery, the animals were housed in separate cages and allowed to adapt to the new environment for 10 days. The animals were fed Ralston Purina guinea pig chow *ad libitum* until 12 hours before injury, at which time the animals were fasted. Before injury, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). Their hair was clipped, and resuscitation fluid was administered intraperitoneally (25 ml of Lactated Ringer's solution/kg body weight). Fourteen of the animals received scald burns on the dorsum and abdomen totalling approximately 25% of the body surface. The other six animals were not burned and served as the controls. The percent of burn injury was calculated by dividing the area burned by the estimated total body surface (37) and multiplying by 100; the time of burning on the dorsum and abdomen was 10 seconds and 4 seconds respectively. After burning or sham-burning, the animals were replaced in their cages and fed *ad libitum*. All animals received a second intraperitoneal injection of Lactated Ringer's solution (25 ml/kg body weight) 8 hours following the procedure. Five of the 14 burned animals and one of the controls did not survive the procedure. The remaining nine burned animals and five controls survived to 30 days postburn, at which time they were euthanized by intraperitoneal injection of T-61 euthanasia solution (1 ml/kg body weight).

Our next experiments were performed using an additional 13 animals. Ten of the animals were burned, and three served as controls. The procedure described above was followed with the addition that the burned animals were placed on heating blankets for 4 hours following injury to reduce the stress associated with heat loss. This modification of the procedure appeared to increase survival; only two of the ten animals did not survive. Twenty-four hours following injury, sections of heart, kidney, liver, spleen, lung, gastrointestinal tract, and skin (dorsum and abdomen) from three of the burned animals and the three controls were prepared for histological examination. The skin from the burned animals was very similar. The burn damage to the dorsum extended approximately two-thirds of the way through the dermis. The burn damage to the abdomen involved the majority of the dermis. No significant abnormalities in the organs of the burned animals or controls were noted.

After performing several additional pilot experiments, the following modifications to increase survival and achieve full-thickness injuries were adopted: (1) the maximal amount of burn injury was limited to approximately 30% of the total body surface of the animals; (2) scald burns were applied only to the dorsal surfaces of the animals rather than to the dorsum and abdomen using a custom-made insulated mold with an exposed area of 60 sq cm (one or two separate burns were required to produce approximately

15% or 30% total body surface burns respectively); (3) the time of burning was increased to 13 seconds; (4) animals with 30% total body surface burns received 50 ml/kg of Lactated Ringer's solution immediately before injury and at 1.5 and 5 hours after injury; (5) animals with 15% total body surface burns received 25 ml/kg of Lactated Ringer's solution immediately before injury and 3 hours after injury; (6) all animals were placed on heating blankets for 6 to 7 hours following injury; and (7) animals were not fasted prior to burn injury.

b. Changes in complement and opsonic activity following experimental burn injury and their relationships to burn size, change in weight, burn wound colonization, and bacteremia

Three groups consisting of 110 male and female Hartley guinea pigs per group were purchased from Murphy Breeding Labs, Inc., at separate times during a 1-year period. Ninety-five percent of the animals survived the 10-day period of acclimation to the new environment. Two of the groups received one or two dorsal burns respectively, and the third group served as the controls. This group was handled identically to the group receiving one dorsal burn, with the exception that the animals were immersed in tepid water and received the second dose of Lactated Ringer's solution on the morning following the procedure. The animals were further subdivided into groups consisting of eight to 11 animals to be sacrificed on the day of burning or sham-burning and at 5-day intervals for 50 days postburn. After anesthesia but before administration of Lactated Ringer's solution and burning or sham-burning, the animals were weighed; the weights ranged from 185 g to 440 g with a mean weight of 326 g. Burn sizes in the groups of burned animals were estimated and found to range from 14.1% to 18.5% (one dorsal burn) and 29.3% to 33.3% (two dorsal burns). The mean burn sizes of the animals in each group are shown in Table 1. Survival on the day following burning or sham-burning was 98%.

The groups of animals sacrificed from 5 to 50 days postburn were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight), weighed, and bled aseptically by cardiac puncture. The groups sacrificed on the day of burning or sham-burning were not reanesthetized or reweighed, since blood was drawn within 2 hours following the procedure; these animals received only one dose of Lactated Ringer's solution given after anesthesia.

Quantitative burn wound cultures were obtained from the burned animals. In addition, autopsies were performed on selected burned animals 24 hours following injury. The results demonstrated that full-thickness injuries had been produced without damage to the visceral organs. Immediately following the procurement of specimens, all animals were euthanized by intraperitoneal injection of T-61 euthanasia solution (1 ml/kg body weight).

Blood was used for preparation of blood cultures and sera. One ml was inoculated into 5 ml of brain heart infusion broth, and the cultures were incubated at 35° C. After 24 and 48 hours of incubation, the cultures were Gram stained and streaked on sheep blood agar and Sabouraud dextrose agar. The plates were incubated for 24 hours at 35° C, and all colonies

Table 1. Estimated Burn Sizes of the Animals.

<u>Proposed Time of Sacrifice in Days Postburn</u>	<u>Mean Burn Size (%)</u>	
	<u>Group 1^a</u>	<u>Group 2^b</u>
0	14.8	30.1
5	15.8	31.0
10	16.1	31.8
15	16.2	31.7
20	16.5	31.1
25	16.6	31.6
30	15.9	32.6
35	16.5	31.9
40	16.5	30.9
45	16.2	30.6
50	15.9	31.2

^aAnimals received one dorsal burn.

^bAnimals received two dorsal burns.

were identified by standard methods (38). The remaining blood was allowed to clot in glass tubes for 1 hour at room temperature and up to 4 hours at 4° C. The tubes were centrifuged at 5,000 x g for 10 minutes at 4° C, and the serum was removed. The serum was divided into small aliquots and frozen at -70° C.

The procedure for obtaining and processing quantitative burn wound cultures described by Saymen et al. was used (39). Burn eschar was incised, and a small specimen of panniculus muscle at the center of the burn wound was removed by sharp dissection. In cases of muscle destruction, subcutaneous tissue was removed. The specimens were transferred aseptically to pre-weighed sterile plastic tubes and weighed. One ml of sterile physiological saline, pH 7.0, was added to each tube in order to transfer the specimen to a 30 ml Potter Elvehjem tissue grinder. The tube was rinsed with 1 ml of sterile saline into the tissue grinder. The loaded grinder was immersed in an ice bath, and the tissue was homogenized for 10 to 15 minutes using a drill press. The total number of bacteria in each specimen was determined by a standard dilution plate count method using sheep blood agar as the growth medium. After 24 hours of incubation at 35° C, individual colony types were counted and Gram stained. The bacteria were identified by standard methods (38). The number of colony forming units (cfu) of individual bacterial species in each specimen was estimated by multiplying the proportion of that species as assessed by colonial morphology by the total number of cfu. Results were expressed as log₁₀ cfu per 100 mg of specimen.

As indicated previously, the sham-burned and burned animals were studied at separate times. Specimens were obtained from the sham-burned animals first and then from the animals with two dorsal burns. The last group studied was that with one dorsal burn. Survival of animals in each group was as follows: (a) 98%, sham-burned; (b) 84%, two dorsal burns; and (c) 96%, one dorsal burn. Mortality of animals with two dorsal burns occurred between 15 and 50 days postburn, and mortality of animals with one dorsal burn occurred between 20 and 40 days postburn. Blood was successfully obtained from 94% of surviving animals. Quantitative burn wound cultures were obtained from 93% of the burned animals.

Total hemolytic complement and alternative pathway-mediated complement consumption were measured in the sera. Total hemolytic complement was titrated by the method of Kabat and Mayer (40) using a volumetric modification. For measurement of alternative pathway-mediated complement consumption, the sera were treated with ethylene glycol-bis- (β-aminoethyl ether) N, N'-tetraacetic acid (EGTA) to block classical pathway activity and leave alternative pathway activity intact. Sera were treated with 10 mM EGTA and incubated for 5 minutes at 37° C (41). Fifty-five and one-half ul of inulin (100 mg/ml) were added to 500 ul of EGTA-treated serum in 12 x 75 mm polystyrene tubes. Physiological saline, pH 7.0, was substituted for inulin in the controls. The tubes were incubated at 37° C, and 111 ul samples were removed at 0 time and at various times to be specified subsequently. The samples were centrifuged at 1,600 x g for 5 minutes at 4° C, and residual total hemolytic complement was titrated in the supernatants as described above. The results were expressed in percent of complement consumption and were calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to total hemolytic complement at 0 time and b was equal to total hemolytic complement at the specified time interval of incubation.

The method of Root et al. (42) was adapted for measurement of opsonic activity in guinea pig sera. Two bacterial test strains and two serum concentrations (10% and 98%) were used. Sera were not tested at a concentration of 50%, because the technique selected for measurement of opsonic activity was much more consuming of time and materials than that proposed in our original application which in preliminary experiments was not found to be satisfactory. Stock cultures of *E. coli* 075 and *S. aureus* 502A were maintained in trypticase soy broth at -70°C . Five μl of thawed culture were inoculated into 1 ml of trypticase soy broth containing 10 $\mu\text{Ci/ml}$ of ^3H -thymidine and incubated for 16 hours at 37°C . Bacteria were pelleted at $5,000 \times g$ for 10 minutes at 4°C , washed once, and suspended in Hank's balanced salt solution containing calcium and magnesium ions and 0.1% gelatin (GHBSS) to a final concentration of 2.5×10^9 cfu/ml. Incorporation in counts per minute (cpm) per 5.0×10^7 cfu ranged from $2.3\text{--}3.6 \times 10^4$ (*E. coli* 075) and $3.6\text{--}9.4 \times 10^4$ (*S. aureus* 502A). Peritoneal exudates were induced in non-burned 300 g Hartley guinea pigs by injection of 15 to 20 ml of 12% casein in sterile physiological saline, pH 7.4, and collected 18 to 20 hours later by lavage with 50 to 70 ml of GHBSS containing 10 units/ml of heparin. The leukocytes were washed twice and suspended in GHBSS to a final concentration of 5.0×10^7 cells/ml. The leukocyte suspensions contained 89% to 92% polymorphonuclear neutrophils and 8% to 11% mononuclear cells. Five hundred μl of leukocyte suspension containing 2.5×10^7 cells were added to 12 x 75 mm capped polyethylene tubes, and the tubes were centrifuged at $225 \times g$ for 7 minutes at 4°C . The supernatants were discarded, and 50 μl or 490 μl of test serum, 10 μl of bacterial suspension (2.5×10^7 cfu), and GHBSS were added to the leukocyte pellets in a final volume of 500 μl . Controls consisting of serum and bacteria were prepared in tubes lacking leukocytes, and controls consisting of leukocytes and bacteria were prepared by substitution of GHBSS for the serum. The tubes were gently vortexed and then tumbled at 37°C on a rotating platform. Immediately after the reaction tubes were constituted and at various time intervals to be specified subsequently, aliquots (100 μl) were removed to 3 ml of GHBSS containing 0.01 M sodium fluoride. Leukocyte-associated bacteria were separated from non-associated bacteria by centrifugation at $120 \times g$ for 5 minutes at 4°C . The leukocyte pellets were washed three times with 3 ml of GHBSS containing sodium fluoride. The final leukocyte pellets were solubilized in 500 μl of NCS tissue solubilizer for 40 minutes at 50°C . Glacial acetic acid (17 μl) was added, and the samples were transferred to glass mini-scintillation vials. A 5 ml volume of organic counting scintillant was added, and the samples were counted in a Beckman LS 7000 liquid scintillation counter. Samples (100 μl) were also removed at the specified time periods from controls lacking leukocytes. The samples were centrifuged at $12,000 \times g$ for 20 minutes at 4°C to deposit the bacteria for measurement of total counts. The pellets were solubilized and counted as described above. The percent of uptake was calculated by dividing the cpm in the leukocyte pellets by the cpm in the corresponding bacterial pellets and then multiplying by 100.

Analysis of variance was used to determine differences in the overall data from the three groups of animals and differences in the data for each postburn day (43). Duncan's multiple range test was used to further describe the differences (44). To determine associations between two variables independent of postburn day and treatment group, partial correlation coefficients were computed (45). Results from the assays of

complement consumption and opsonic activity and the quantitative burn wound culture results were transformed before computing analysis of variance and partial correlation coefficients. The transformations that were used were as follows: (a) arcsin (complement consumption), (b) \log_e (opsonic activity), and (c) square root (quantitative cultures) (46). A probability value of less than 0.05 was considered to be significant in all tests.

Initial experiments were performed to determine the optimal time intervals of incubation for measurement of alternative pathway-mediated complement consumption and opsonic activity. Complement consumption in EGTA-treated pooled normal guinea pig serum by inulin was 55% by 15 minutes of incubation and increased to 76% by 45 minutes (Figure 1). Complement consumption was negligible during 45 minutes of incubation of the serum with saline. Uptake by guinea pig peritoneal PMNL of *E. coli* 075 and *S. aureus* 502A in the presence of 10% or 98% of pooled normal guinea pig serum was maximal at 10 minutes of incubation (Figure 2). Uptake of *E. coli* 075 was negligible in the absence serum. Uptake of *S. aureus* 502A under these conditions was demonstrated; however, the kinetics of uptake were decreased as compared with the kinetics observed in the presence of serum. The time interval of incubation that was selected for measurement of complement consumption and opsonic activity in our subsequent experiments was 10 minutes.

The next data that will be presented were obtained from determinations of complement and opsonic activity on sera from the two groups of burned animals and the sham-burned controls. These determinations were performed during an 18-month period. Because of possible variability in the assays over time, the sera were tested in a randomized fashion; appropriate positive and negative controls were included in all experiments.

Total hemolytic complement was significantly increased in both groups of burned animals as compared with the controls beginning 5 days postburn and continuing through 35 days postburn (Figure 3). In the animals with larger burns, total hemolytic complement peaked on day 20 and decreased to normal or near-normal levels after day 35. In the animals with smaller burns, a similar pattern was observed through day 30, although total hemolytic complement was significantly decreased on days 25 and 30 as compared with total hemolytic complement in the animals with larger burns. After day 30, fluctuation was observed with total hemolytic complement being significantly increased on day 40 as compared with total hemolytic complement in the animals with larger burns. This latter observation is of questionable biological significance and is probably related to the wide variation of results observed on day 40.

Alternative pathway-mediated complement consumption by inulin was decreased from 10 through 45 days postburn in the animals with larger burns as compared with the control animals and the animals with smaller burns (Figure 4). Analysis of variance on the overall data from the three groups of animals showed that the decrease in the animals with larger burns was significant. Analysis of variance by day revealed a significant difference at 35 days postburn (the nadir for the animals with larger burns). A significant correlation between alternative pathway-mediated complement consumption by inulin and total hemolytic complement was not demonstrated.

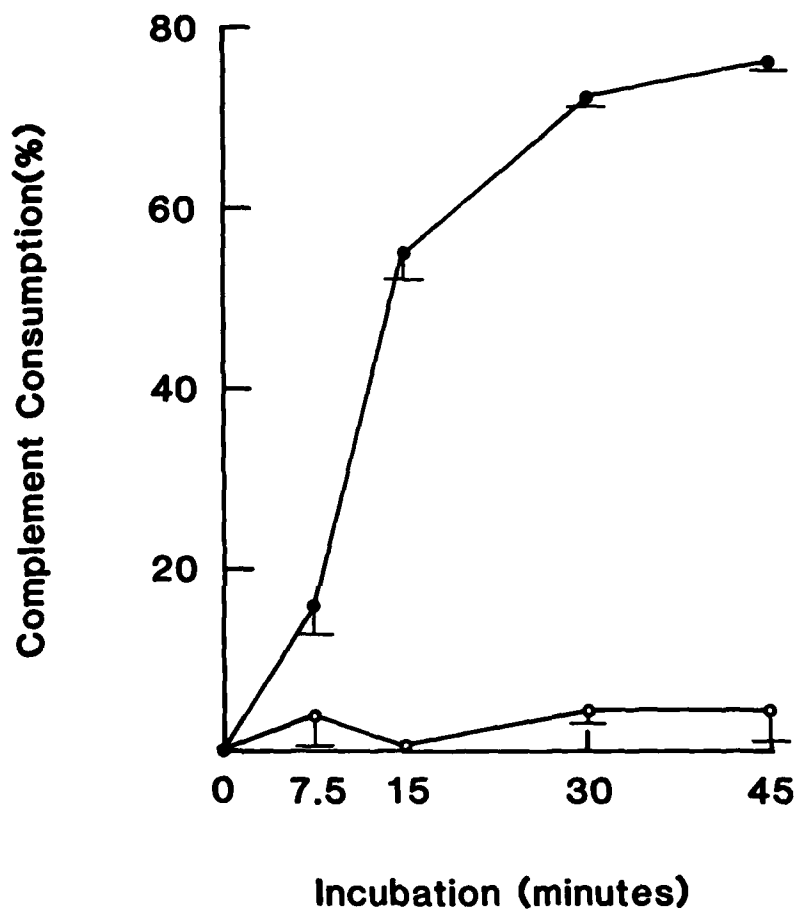


Figure 1. Kinetics of complement consumption in EGTA-treated pooled normal guinea pig serum during incubation with inulin (•) or saline (o). The points represent average values of two determinations, and each vertical bar represents the standard error of the mean.

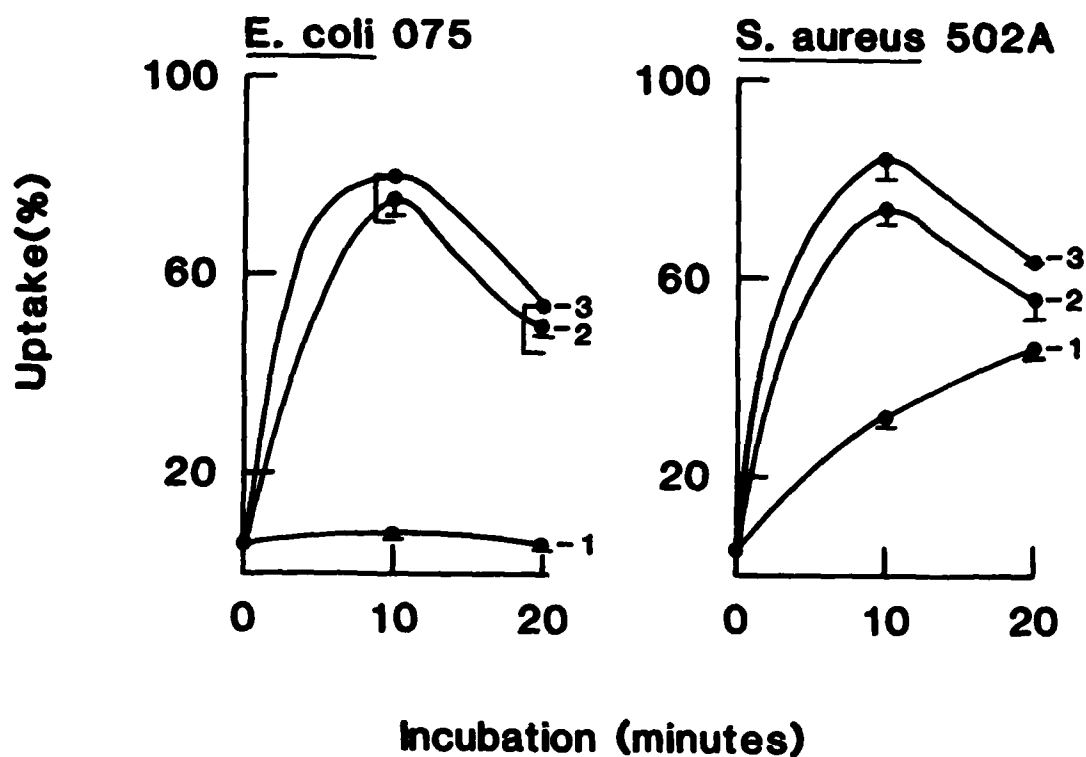


Figure 2. Kinetics of uptake of *E. coli* 075 and *S. aureus* 502A by guinea pig peritoneal PMNL in the presence of pooled normal guinea pig serum. Reaction mixtures consisted of bacteria and the following reactants: (1) PMNL, (2) PMNL and 10% of serum, and (3) PMNL and 98% of serum. The points represent average values of two determinations, and each vertical bar represents the standard error of the mean.

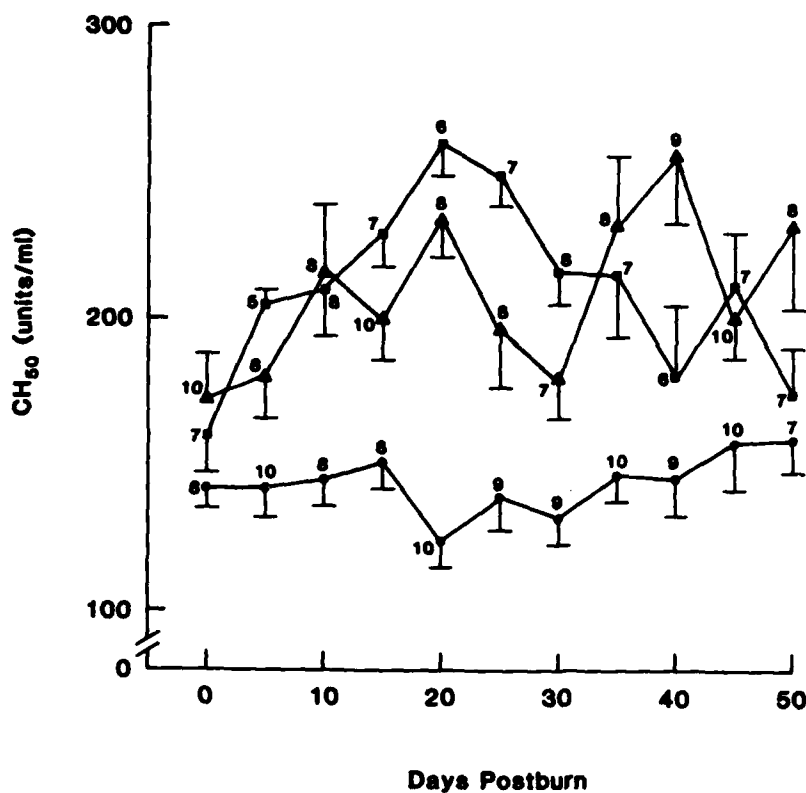


Figure 3. Total hemolytic complement in the sera of guinea pigs during 50 days following sham-burning (●) or burning of approximately 15% (▲) or 30% (■) of the total body surface. The points represent mean values, and each vertical bar represents the standard error of the mean. The numbers at each point specify animals from which sera were analyzed.

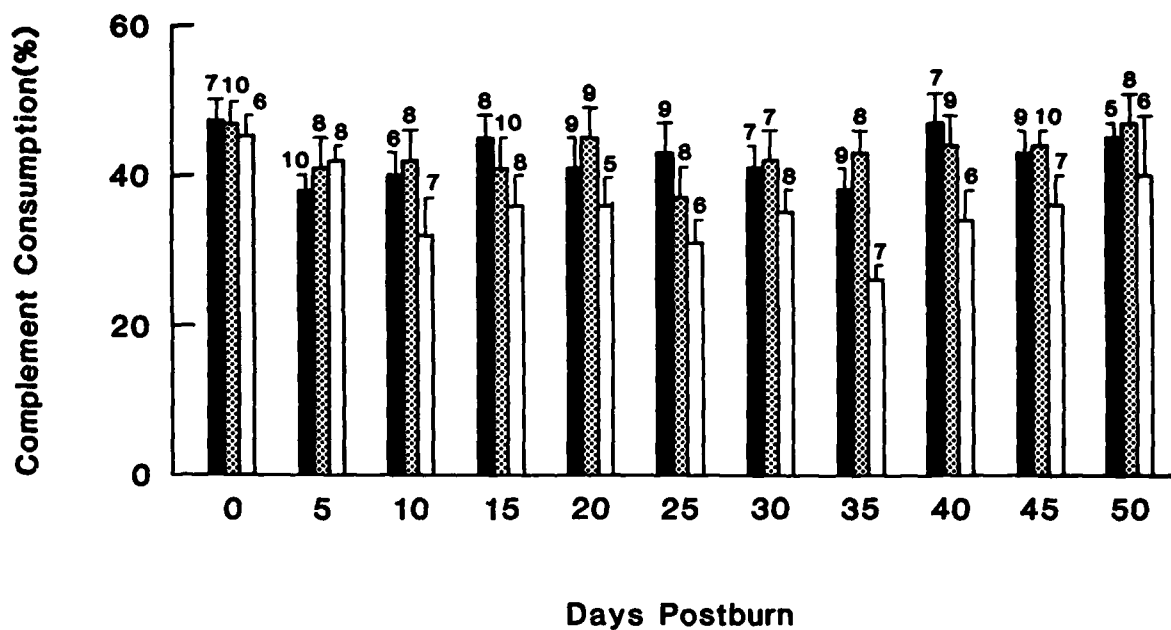


Figure 4. Complement consumption by inulin in EGTA-treated sera from animals with approximately 15% (▨) and 30% (□) total body surface burns and sham-burned animals (■). The mean, standard error of the mean, and number of animals at each day postburn are shown.

Sera from the animals with larger burns and the controls facilitated equivalent uptake of E. coli 075 and S. aureus 502A by normal guinea pig peritoneal PMNL when tested at a concentration of 10% (Figures 5 and 6). When the sera were tested at a concentration of 98%, diminution in opsonic activity in the burned animals as compared with the controls was demonstrated from 10 through 25 days postburn using E. coli 075 but not S. aureus 502A as the test strain (Figures 7 and 8). Analysis of variance revealed a significant difference in the overall data from the two groups of animals and the data obtained on day 15 (Figure 7). The difference observed on day 15 was further investigated by measuring the kinetics of uptake of E. coli 075 in the presence of 98% of sera from six animals in each group. The results showed that the kinetics of uptake in the presence of sera from the burned animals were decreased as compared with the kinetics observed in the presence of the control sera; the differences approached significance ($p=0.06, 0.055$, and 0.041 at 5, 10, and 20 minutes respectively) (Figure 9). Significant correlations between opsonic activity for E. coli 075 (98% serum concentration) and total hemolytic complement or alternative pathway-mediated complement consumption were not demonstrated.

Opsonic activity for E. coli 075 in sera obtained on day 15 from ten animals with smaller burns has also been measured using the 98% serum concentration. Mean uptake \pm the standard error of the mean was $82.4\% \pm 1.28\%$, which was equivalent to uptake in the presence of the control sera. Additional determinations of opsonic activity in sera from animals with smaller burns have not yet been performed.

Change in weight during 50 days following burning or sham-burning is shown in Figure 10. Weight loss was documented during 20 days postburn in the animals with larger burns. Maximal weight loss occurred 10 days following burn injury and was 9% of the pre-burn weight. The weights of the animals with smaller burns remained relatively constant during 5 days postburn and increased thereafter. Weight gain was observed in the control animals at 5 days following sham-burning and on all subsequent days. The changes in weight in the animals with larger burns differed significantly from the controls at all days postburn. In the animals with smaller burns, change in weight differed significantly from the controls at all days except day 5. Significant differences in weight change in the two burned groups were demonstrated at all days except days 5, 15, and 30. Significant correlations between change in weight and total hemolytic complement, alternative pathway-mediated complement consumption, or opsonic activity for E. coli 075 (98% serum concentration) were not demonstrated.

The time course of colonization of the burn wounds in the two groups of burned animals was similar (Figure 11). Immediately following burn injury, few bacteria were isolated from burn wound tissue (0-250 cfu per 100 mg). By 10 days postburn, all animals in both burned groups were colonized; cfu per 100 mg of burn wound tissue ranged from 2.0×10^5 to 9.0×10^7 . Colonization persisted in both groups of animals during 50 days postburn. At days 10 and 20, the number of bacteria isolated from the burn wounds of the animals with larger burns was significantly greater than the number of bacteria isolated from the animals with smaller burns. The most common infecting agent was S. aureus; other isolates were Staphylococcus epidermidis, alpha streptococci, Acinetobacter calcoaceticus, Proteus vulgaris, E. coli, Pseudomonas aeruginosa, Aeromonas hydrophila, Citrobacter species, Shigella species, and Bacillus species.

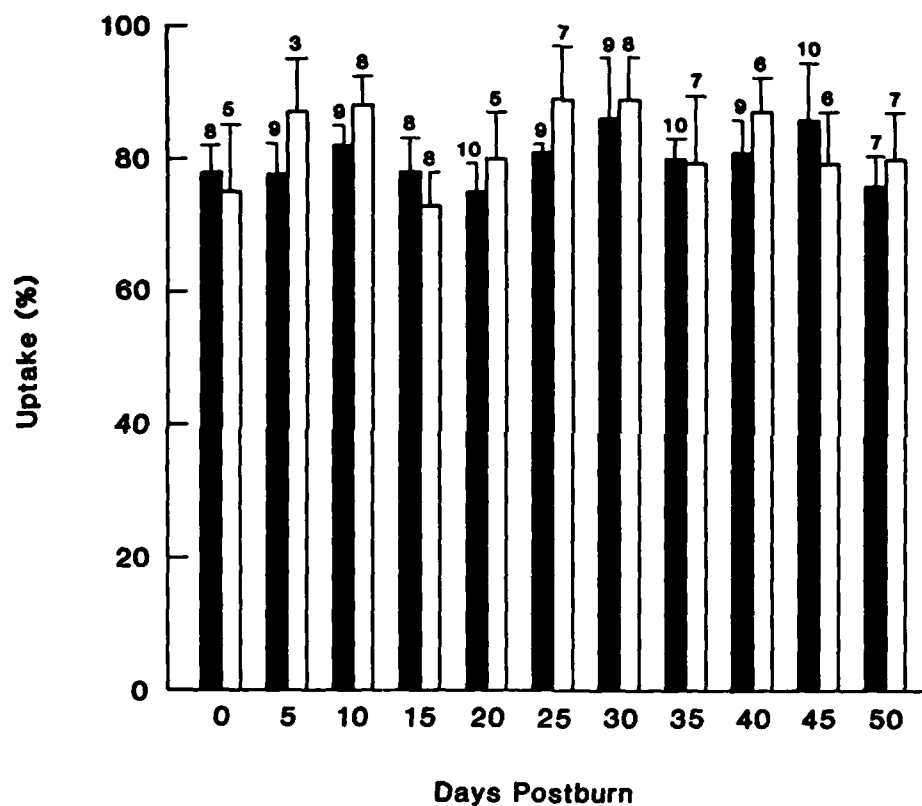


Figure 5. Uptake of *E. coli* 075 by normal guinea pig peritoneal PMNL in the presence of 10% of sera from animals with approximately 30% total body surface burns (□) and sham-burned animals (■). The mean, standard error of the mean, and number of animals at each day postburn are shown.

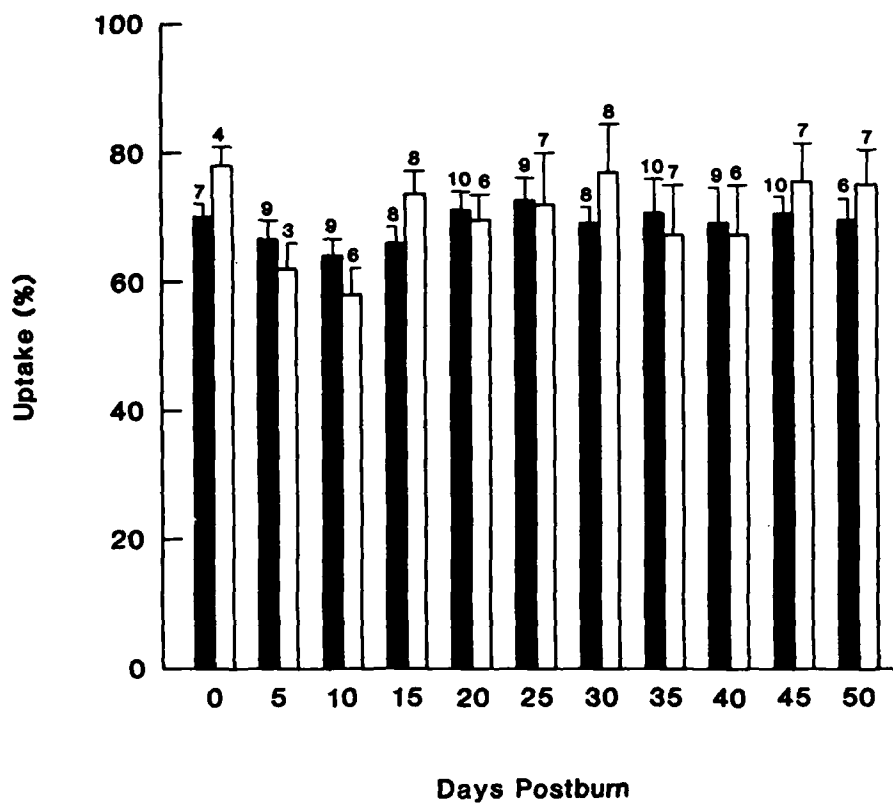


Figure 6. Uptake of *S. aureus* 502A by normal guinea pig peritoneal PMNL in the presence of 10% of sera from animals with approximately 30% total body surface burns (□) and sham-burned animals (■). The mean, standard error of the mean, and number of animals at each day postburn are shown.

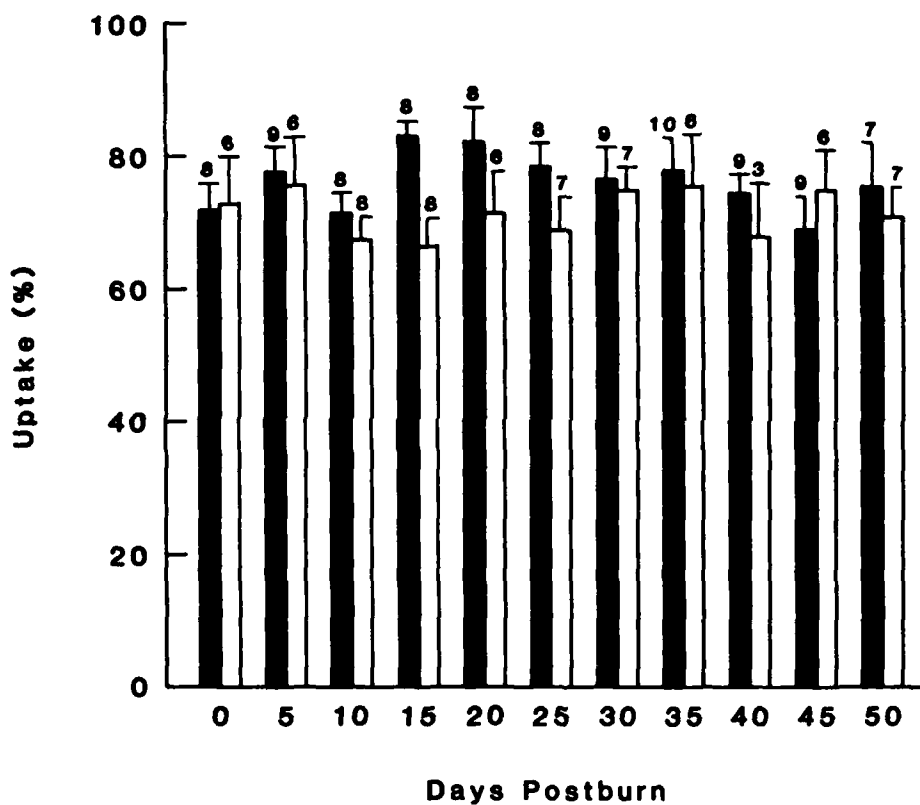


Figure 7. Uptake of *E. coli* 075 by normal guinea pig peritoneal PMNL in the presence of 98% of sera from animals with approximately 30% total body surface burns (□) and sham-burned animals (■). The mean, standard error of the mean, and number of animals at each day postburn are shown.

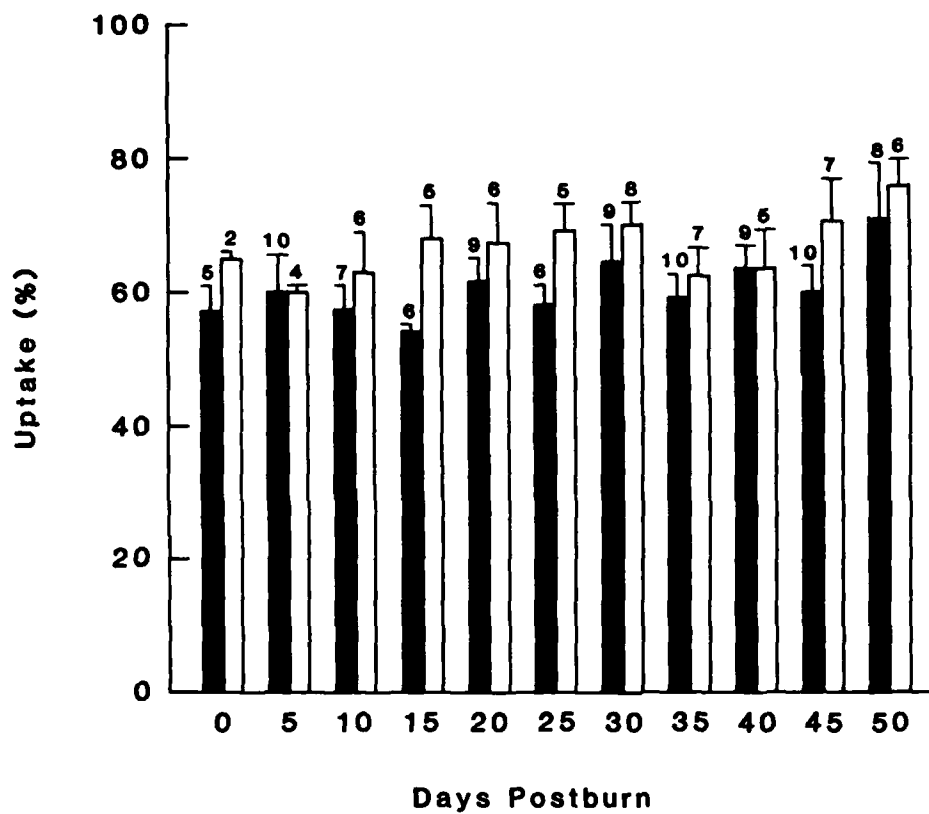


Figure 8. Uptake of *S. aureus* 502A by normal guinea pig peritoneal PMNL in the presence of 98% of sera from animals with approximately 30% total body surface burns (□) and sham-burned animals (■). The mean, standard error of the mean, and number of animals at each day postburn are shown.

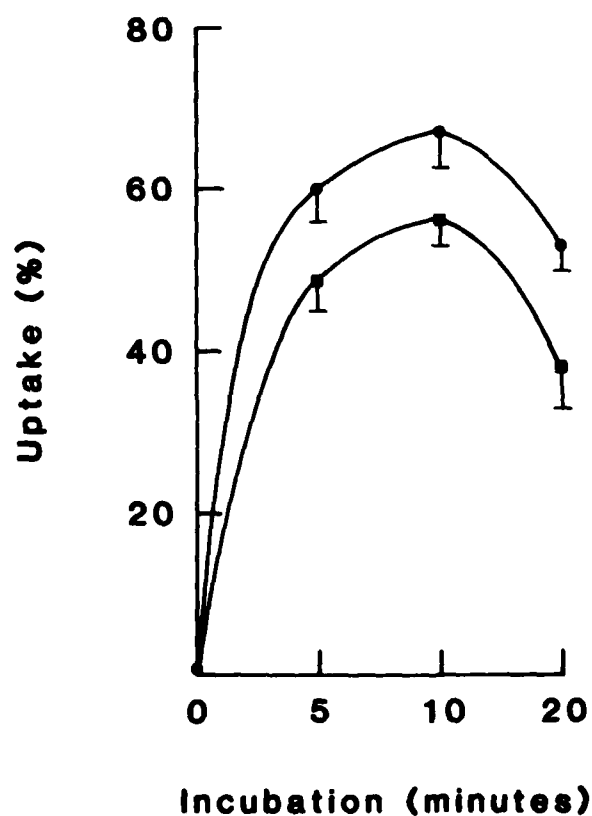


Figure 9. Kinetics of uptake of *E. coli* 075 by normal guinea pig peritoneal PMNL in the presence of 98% of sera obtained on day 15 from six animals with approximately 30% total body surface burns (■) and six sham-burned animals (●). The points represent mean values, and each vertical bar represents the standard error of the mean.

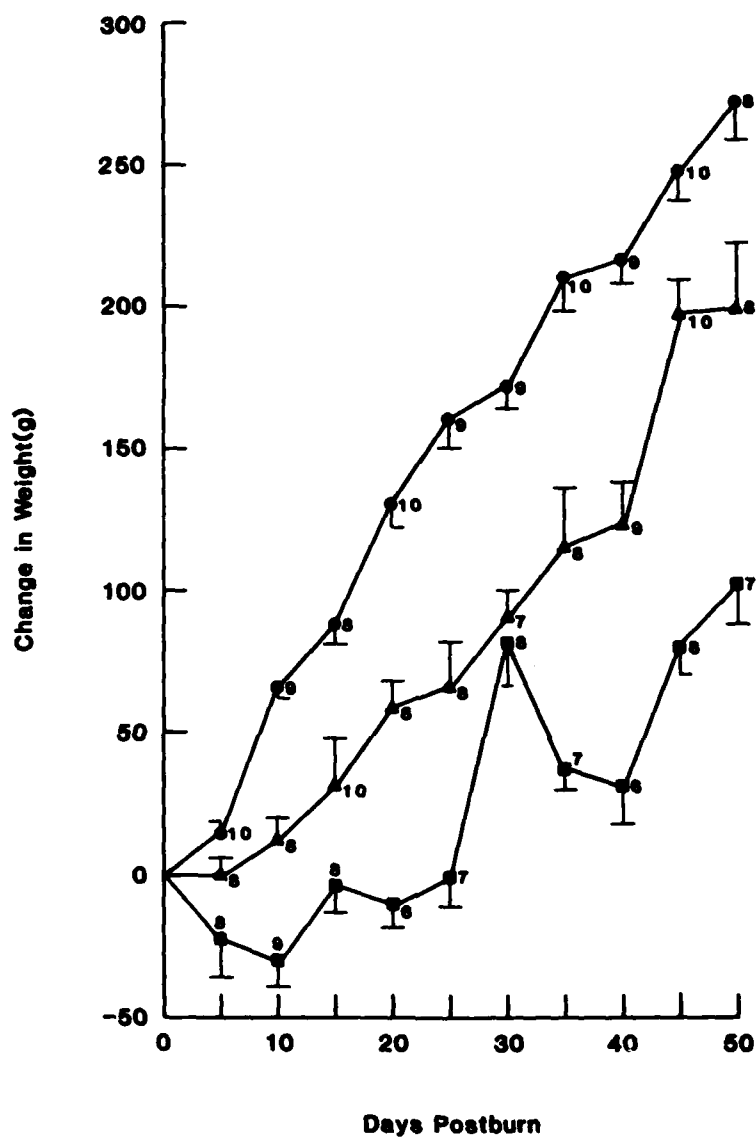


Figure 10. Change in weight during 50 days following sham-burning (●) or burning of approximately 15% (▲) or 30% (■) of the total body surface. The points represent mean values, and each vertical bar represents the standard error of the mean. The numbers at each point specify animals from which weights were measured.

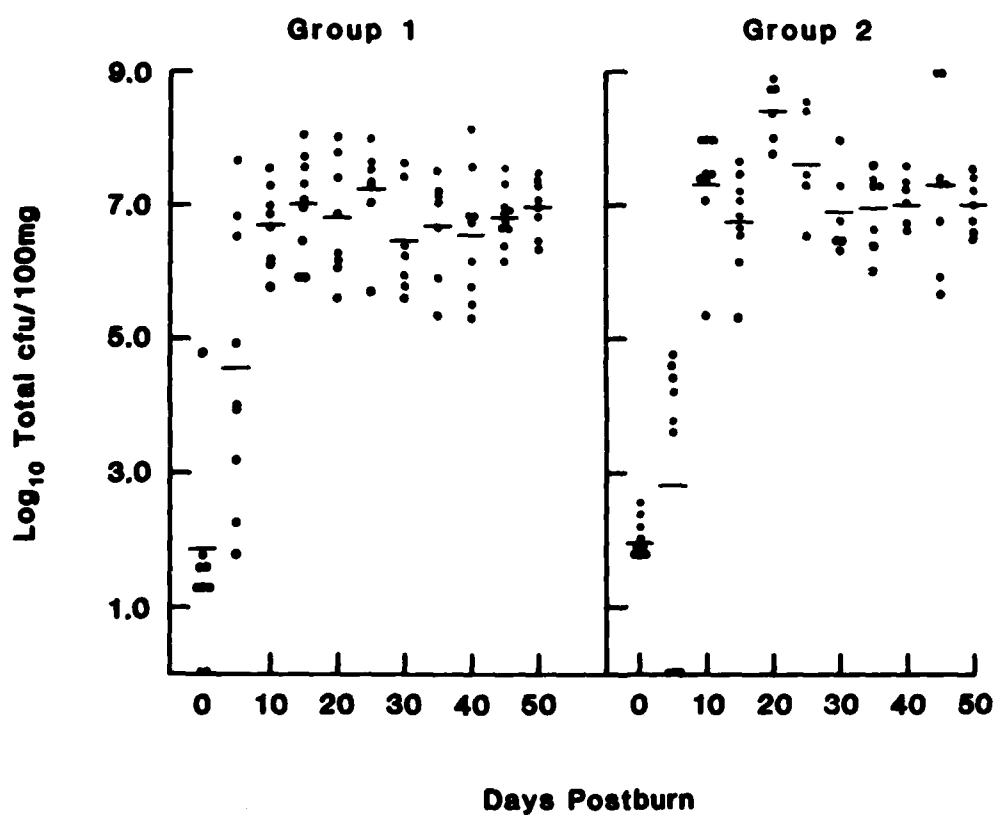


Figure 11. Total cfu per 100 mg of burn wound tissue in animals with approximately 15% (Group 1) and 30% (Group 2) total body surface burns. The points represent the values obtained from all animals, and the horizontal lines represent mean values for each day postburn.

The frequency of isolation of Gram-positive cocci from the two groups of burned animals was similar except on day 5, when a greater frequency of isolation was noted in the animals with smaller burns (Figure 12). Comparable numbers of these bacteria were isolated from the two groups of animals after day 5. Similar results were obtained when the data were analyzed according to the frequency of isolation and the number of S. aureus isolated from the two groups of animals, with the exception that after day 5 S. aureus was isolated with greater frequency from the animals with larger burns (Figure 13).

The frequency of isolation of Gram-negative rods from the two burned groups was quite different (Figure 14). On day 10, these bacteria were isolated with greater frequency from the animals with smaller burns, but by day 20 only one of eight of these animals had positive cultures as compared with five of six animals with larger burns. After day 20, colonization with Gram-negative rods persisted in 28% of the animals with larger burns but was not observed in the animals with smaller burns.

Significant correlations were not demonstrated between the total number of bacteria per 100 mg of burn wound tissue in the two groups of animals and total hemolytic complement, alternative pathway-mediated complement consumption, or opsonic activity for E. coli 075 (98% serum concentration). Correlations between colonization and the humoral parameters were also not noted, when the quantitative burn wound culture results were analyzed according to numbers of Gram-positive cocci, S. aureus, or Gram-negative rods.

Blood cultures were positive in four of the animals with larger burns and three of the animals with smaller burns; blood cultures were negative in all of the sham-burned controls. Three of the animals with larger burns sacrificed on days 15, 20, and 45 had positive blood cultures for S. aureus, and a fourth sacrificed on day 10 had a positive blood culture for E. coli. Two of the animals with smaller burns sacrificed on days 25 and 50 had positive blood cultures for B. species, and a third sacrificed on day 5 had a positive blood culture for S. aureus and alpha streptococci. This animal had relatively low numbers of bacteria colonizing the burn wound as compared with the other animals; the burn wound isolates from this animal were S. aureus and A. hydrophila (Table 2). Two of the other animals with positive blood cultures for S. aureus had only S. aureus colonizing the burn wound, and the third was colonized with P. vulgaris. (S. aureus colonization in this animal may have been missed because of swarming of P. vulgaris on the agar plates.) E. coli was isolated from the burn wound of the animal with the positive blood culture for E. coli. One of the animals with a positive blood culture for B. species had S. epidermidis colonizing the burn wound, and the other was colonized with S. aureus and alpha streptococci. The latter blood culture results are presumed to represent contamination, since B. species is a frequent inhabitant of skin and hair and was not isolated from the burn wounds.

Total hemolytic complement, alternative pathway-mediated complement consumption, opsonic activity, and change in weight in each bacteremic animal were compared to the mean values for these determinations in that animal's respective postburn day group. Lower alternative pathway-mediated complement consumption was demonstrated in the two animals with larger burns with S. aureus bacteremia and colonization, and lower opsonic activity for E. coli 075 (98% serum concentration) was demonstrated in one of

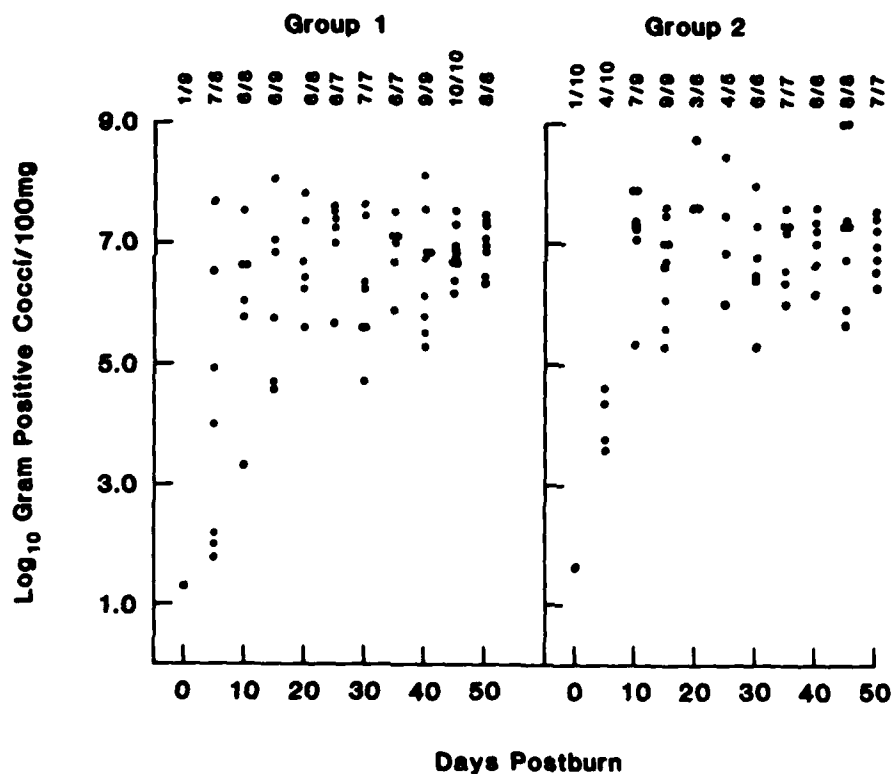


Figure 12. Gram-positive cocci per 100 mg of burn wound tissue in animals with approximately 15% (Group 1) and 30% (Group 2) total body surface burns. The points represent the values obtained on animals with positive cultures, and the designations at the top of the figures represent the number of animals with positive cultures per total animals at each day postburn.

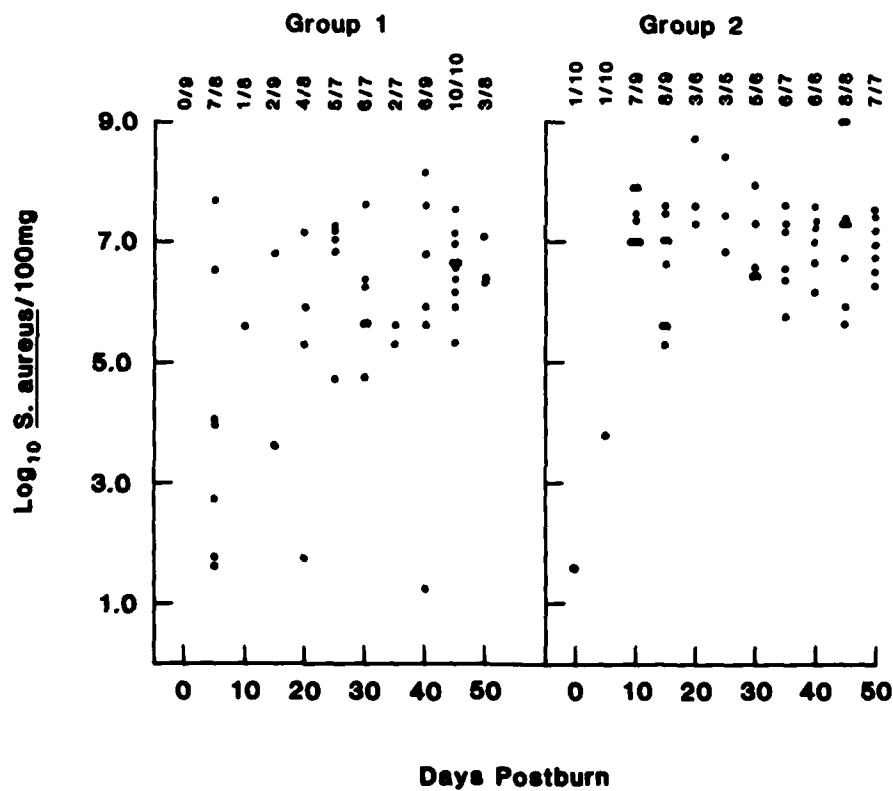


Figure 13. *S. aureus* per 100 mg of burn wound tissue in animals with approximately 15% (Group 1) and 30% (Group 2) total body surface burns. The points represent the values obtained on animals with positive cultures, and the designations at the top of the figures represent the number of animals with positive cultures per total animals at each day postburn.

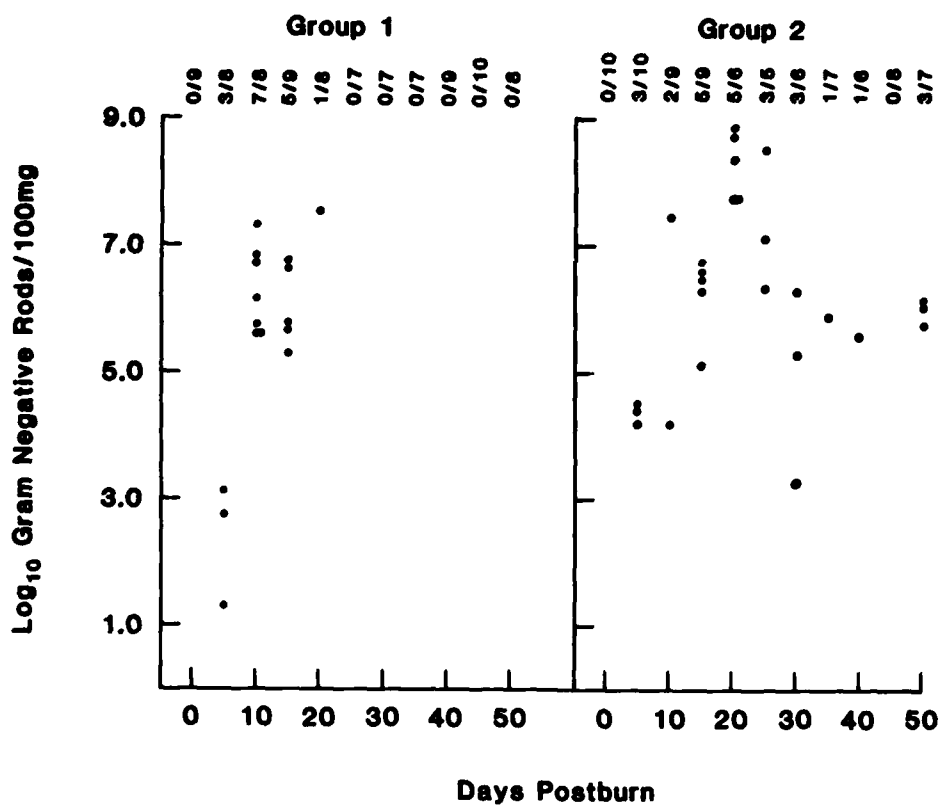


Figure 14. Gram-negative rods per 100 mg of burn wound tissue in animals with approximately 15% (Group 1) and 30% (Group 2) total body surface burns. The points represent the values obtained on animals with positive cultures, and the designations at the top of the figures represent the number of animals with positive cultures per total animals at each day postburn.

Table 2. Quantitative Burn Wound Culture Results in Animals with Positive Blood Cultures.

Days Postburn	Group 1 ^a			Group 2 ^b	
	Blood Culture Isolates	Burn Wound Isolates cfu/100mg	Blood Culture Isolates	Burn Wound Isolates cfu/100mg	
5	<u>S. aureus</u> <u>α-streptococci</u>	8.0 x 10 ³ <u>S. aureus</u> 5.6 x 10 ² <u>A. hydrophila</u>	--	--	
10	--	--	<u>E. coli</u>	2.8 x 10 ⁷ <u>E. coli</u>	
15	--	--	<u>S. aureus</u>	1.2 x 10 ⁷ <u>S. aureus</u>	
20	--	--	<u>S. aureus</u>	5.2 x 10 ⁸ <u>P. vulgaris</u>	
25	<u>B. species</u>	1.95 x 10 ⁷ <u>S. aureus</u> 6.0 x 10 ⁵ <u>α-streptococci</u>	--	--	
45	--	--	<u>S. aureus</u>	2.0 x 10 ⁷ <u>S. aureus</u>	
50	<u>B. species</u>	7.2 x 10 ⁶ <u>S. epidermidis</u>	--	--	

^aAnimals received one dorsal burn.

^bAnimals received two dorsal burns.

these animals (sacrificed on day 15) and in the animal with E. coli bacteremia. Greater weight loss was documented in the three animals with larger burns with bacteremia occurring between days 10 and 20. No other notable differences were observed.

c. Effects of experimental burn wound infection on the humoral parameters

During the project period, a pilot study was also conducted to determine the effects of experimental burn wound infection with S. aureus, P. aeruginosa, and Candida albicans on the various humoral parameters of host defense. Thirty percent total body surface burns were applied to three groups of guinea pigs (12-14 animals per group). Immediately following the procedure, the animals in each group were injected subcutaneously under the burn wound with 5.0×10^5 cfu of S. aureus, P. aeruginosa, or C. albicans; these strains were blood culture isolates from burned humans. Survival at the end of 5 days in each group was as follows: 50% (S. aureus), 33% (P. aeruginosa), and 57% (C. albicans). Surviving animals were sacrificed during 5 to 13 days postburn. The animals were anesthetized and weighed; blood for preparation of blood cultures and sera were collected prior to sacrifice.

Weight loss was documented in all animals (Figure 15). Mean weight loss for each group was as follows: -54 g (S. aureus), -66 g (P. aeruginosa), and -60 g (C. albicans). Blood cultures were positive in only one animal. This animal was experimentally infected with S. aureus, sacrificed on day 5, and had a positive blood culture for S. aureus.

Sera were tested for total hemolytic complement, alternative pathway-mediated complement consumption by inulin, and opsonic activity for E. coli 075 and S. aureus 502A using 10% and 98% serum concentrations. Total hemolytic complement in the sera of two animals infected with S. aureus and one animal infected with C. albicans was decreased as compared with the control values (Figure 16). One of these animals was that with the positive blood culture. Total hemolytic complement in the sera of the majority of other animals fell within the normal range. When the experimental values for each group were compared with the control values, significant differences were not noted. Alternative pathway-mediated complement consumption by inulin in the sera of the experimentally infected animals was markedly reduced as compared with the control values, and significant differences between each group and the controls were demonstrated (Figure 17). Testing of opsonic activity using a serum concentration of 10% revealed different results with the two bacterial test strains (Figures 18 and 19). Although most of the animals had normal or elevated opsonic activity for both strains as compared with the controls, five had decreased opsonic activity for E. coli 075 but not S. aureus 502A; four had decreased opsonic activity for S. aureus 502A but not E. coli 075. Marked reduction in opsonic activity for E. coli 075 was demonstrated, when the sera from all experimentally infected groups were tested at a concentration of 98% (Figure 20). Decreased opsonic activity was detected as early as day 5 and persisted during the 13-day period of study. Diminution in opsonic activity for S. aureus 502A was not demonstrated under these conditions (Figure 21).

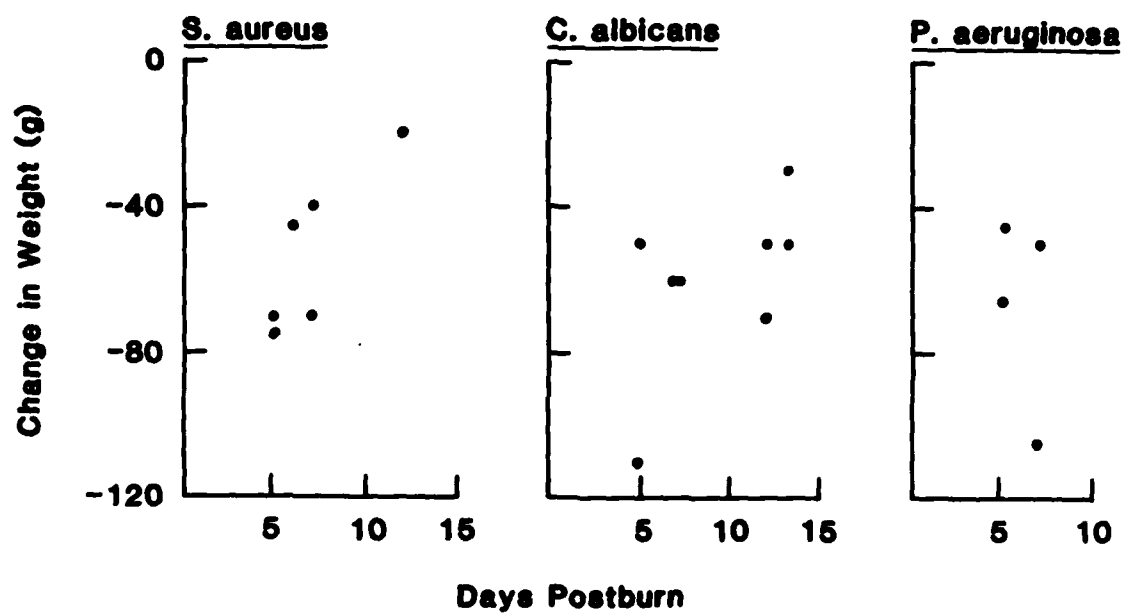


Figure 15. Change in weight in burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*.

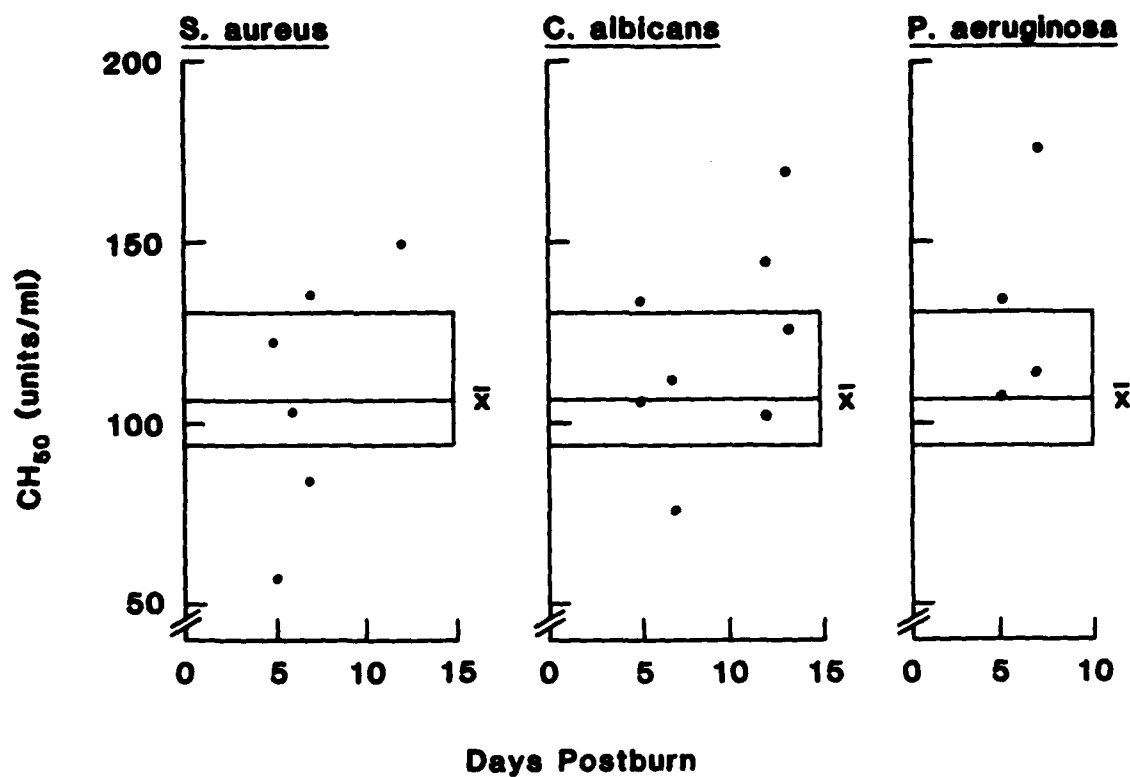


Figure 16. Total hemolytic complement in the sera of burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

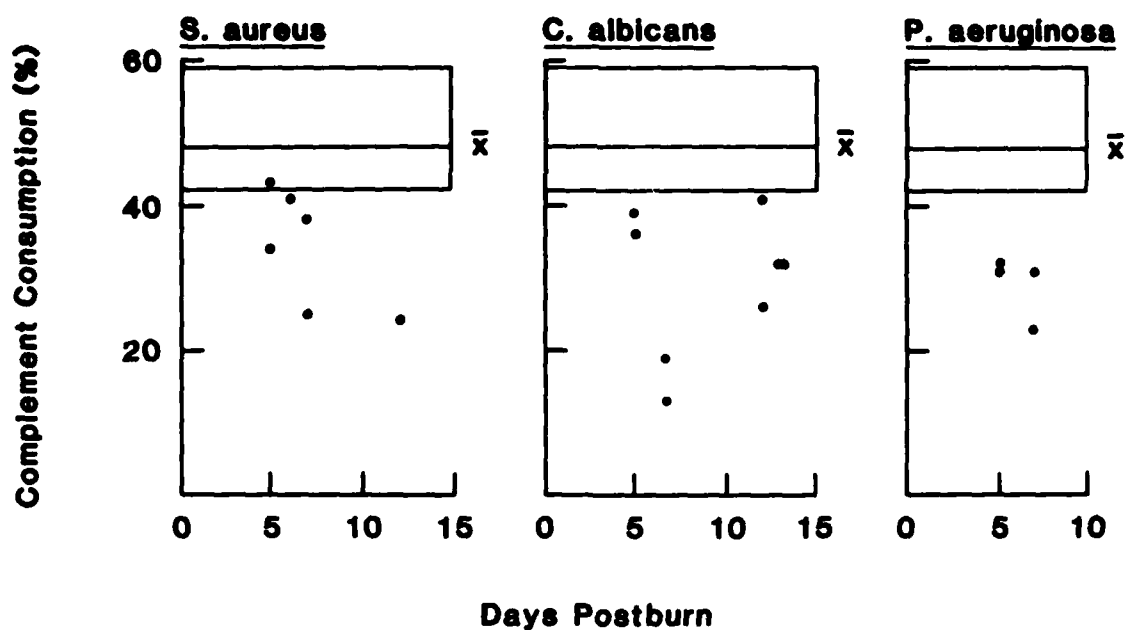


Figure 17. Alternative pathway-mediated complement consumption by inulin in the sera of burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

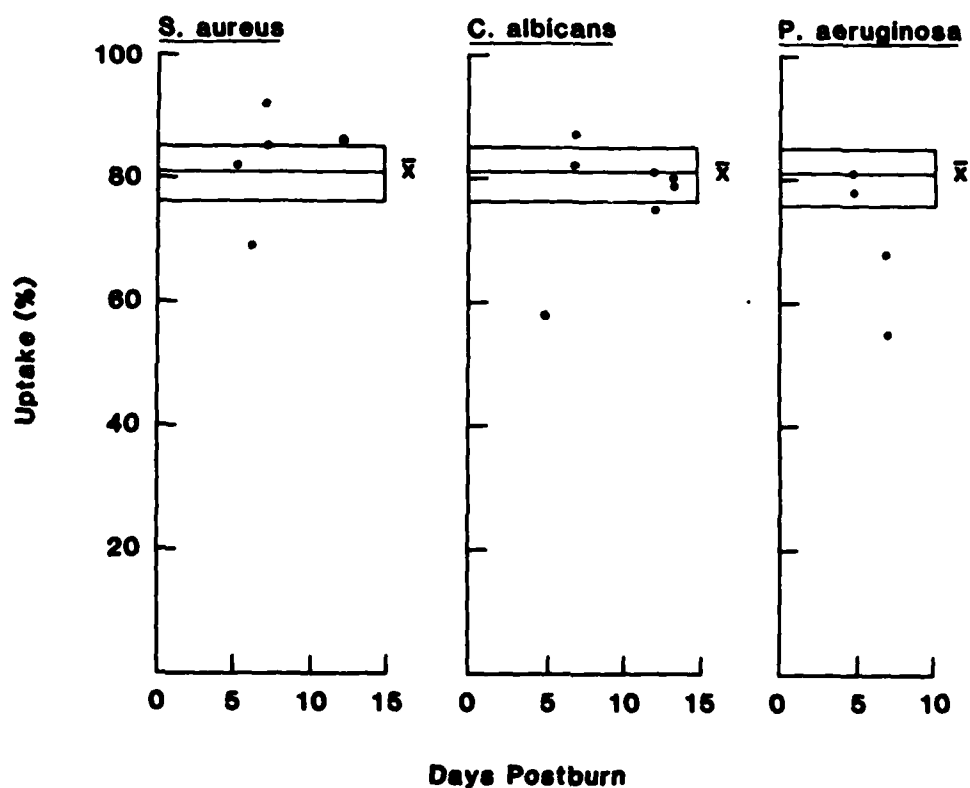


Figure 18. Uptake of *E. coli* 075 by normal guinea pig peritoneal PMNL in the presence of 10% of sera from burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

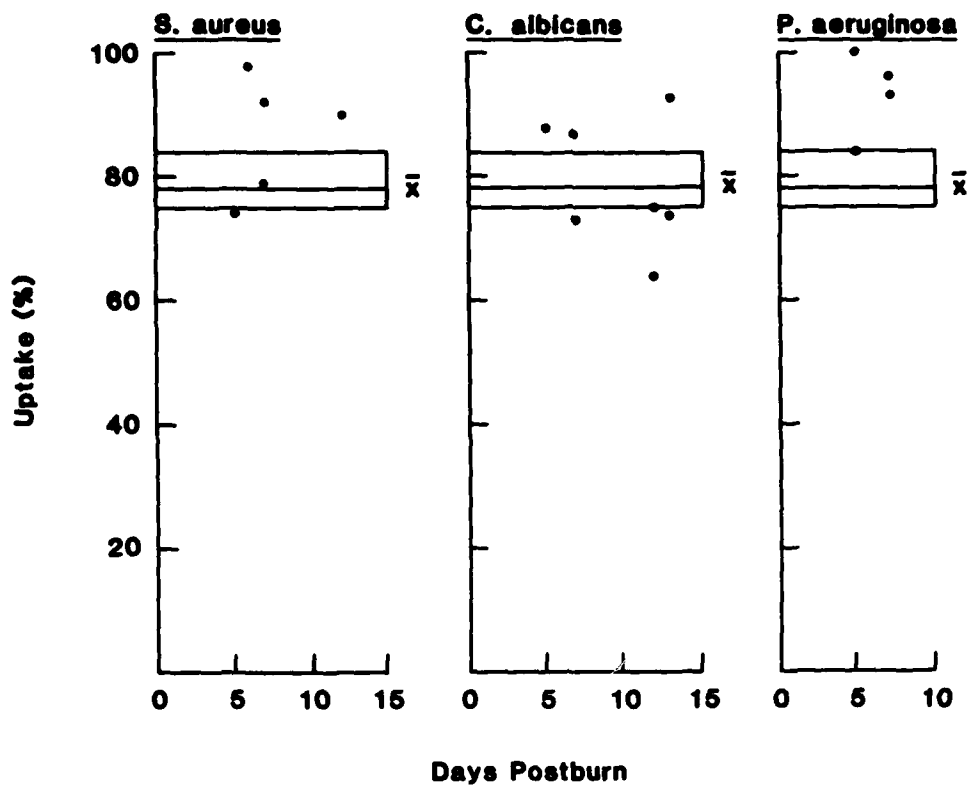


Figure 19. Uptake of *S. aureus* 502A by normal guinea pig peritoneal PMNL in the presence of 10% of sera from burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

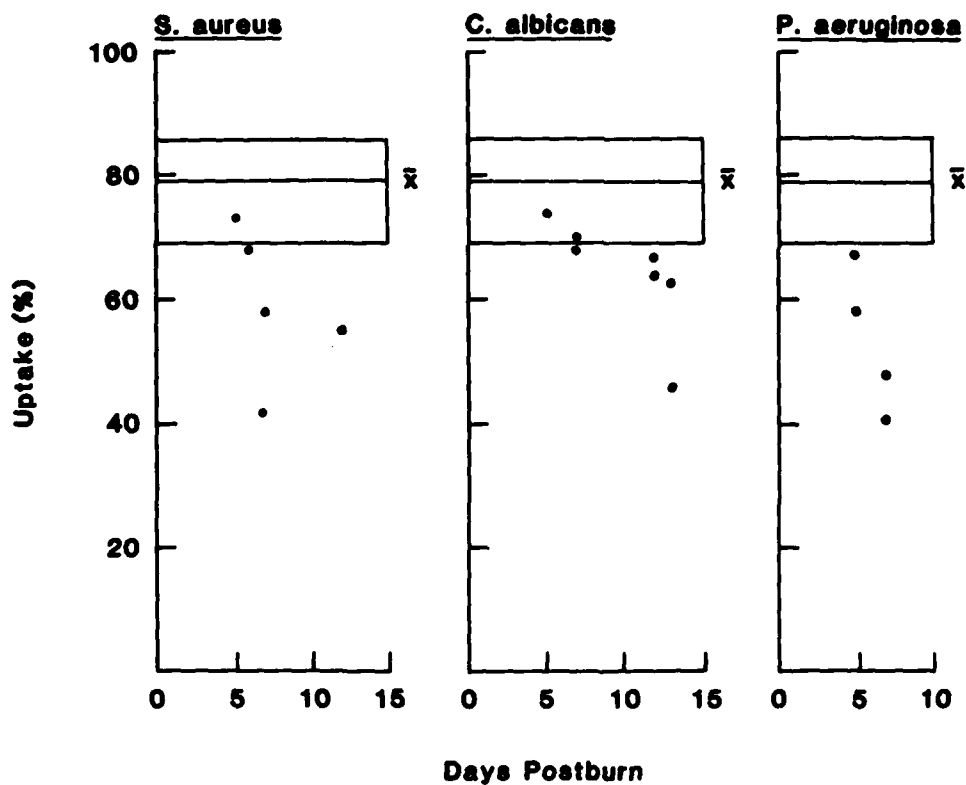


Figure 20. Uptake of *E. coli* 075 by normal guinea pig peritoneal PMNL in the presence of 98% of sera from burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

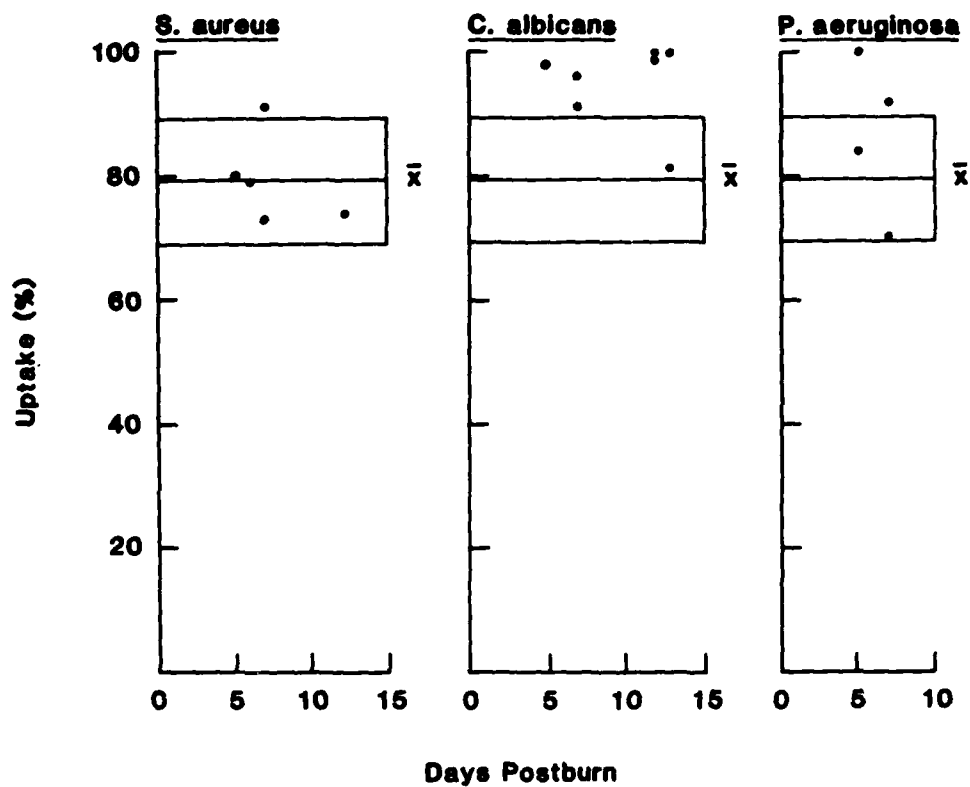


Figure 21. Uptake of *S. aureus* 502A by normal guinea pig peritoneal PMNL in the presence of 98% of sera from burned animals experimentally infected with *S. aureus*, *C. albicans*, and *P. aeruginosa*. The mean and range of values for the control serum (pooled normal guinea pig serum) in the various experiments are shown in the boxed area.

2. Discussion

In our investigation, experimental scald burn injury in guinea pigs was shown to result in elevation of total hemolytic complement, diminution in alternative pathway-mediated complement consumption, and serum-mediated inhibition of bacterial phagocytosis by casein-induced peritoneal PMNL. The latter two abnormalities were demonstrated in animals with 30% total body surface burns; these abnormalities appeared 10 days following burn injury, when weight loss was maximal and burn wound colonization with bacteria was extensive. Reduction in alternative pathway-mediated complement consumption continued through 45 days postburn and was associated with persistent burn wound colonization with aerobic and facultative Gram-negative rods, S. aureus, and other Gram-positive cocci. Serum-mediated inhibition of phagocytosis by PMNL was observed through 25 days postburn. Elevation of total hemolytic complement was demonstrated in animals with 15% and 30% total body surface burns from 5 through 35 days postburn; higher values were observed in the animals with larger burns, suggesting that elevation of total hemolytic complement is related to the extent of burn injury. After day 35, variable results of determinations of total hemolytic complement in the burned animals were observed, and conclusions could not be reached regarding differences between the groups.

Survival of the burned animals during the 50-day period of study was excellent, and bacteremia was observed rarely and did not predominate in any particular postburn day group. Animals with bacteremia not attributed to contamination generally were colonized with the same bacterial strain as was isolated from blood; S. aureus was the most frequent blood culture isolate. Alterations of total hemolytic complement, alternative pathway-mediated complement consumption, and serum phagocytosis-promoting activity in the bacteremic animals were not consistently more pronounced than in non-bacteremic animals. The rare occurrence of positive blood cultures in the test animals probably reflects transient bacteremia. Alternatively, bacteremia may not have been documented accurately, because only one blood culture result was able to be obtained on each animal.

Experimental burn wound infection with S. aureus, P. aeruginosa, and C. albicans in guinea pigs with 30% total body surface burns was associated with reduction in total hemolytic complement, greater serum-mediated inhibition of phagocytosis by PMNL and diminution in alternative pathway-mediated complement consumption, and earlier appearance of these latter abnormalities. Total hemolytic complement in the majority of experimentally infected animals fell within or below the normal range during the 5 to 13 day period of study, and the marked elevation of total hemolytic complement demonstrated in non-experimentally infected animals with similar burn sizes was not observed. Reduction in alternative pathway-mediated complement consumption and serum-mediated inhibition of phagocytosis were demonstrated between 5 to 7 days postburn and persisted in all animals through day 13. Experimental burn wound infection was associated with increased morbidity and mortality, although blood culture results were generally negative. This latter observation is in agreement with the findings of other investigators of the limited value of blood cultures in documentation of invasive burn wound infection (47, 48).

Inhibition of phagocytosis by sera from experimentally infected and non-experimentally infected animals was only demonstrated using E. coli 075 as the bacterial test strain and a physiological concentration of the sera. Failure to detect serum-mediated inhibition of phagocytosis using S. aureus 502A as the test strain may have been related to the finding that this bacterium adhered to the PMNL to some extent in the absence of serum. The assay system might not therefore have been sensitive enough to detect an inhibitory effect, especially if the effect occurred at the level of adherence. Lowering of the serum concentration to 10% resulted in demonstration of normal phagocytosis-promoting activity for both bacterial strains in the majority of sera, suggesting that the sera were not deficient in proteins required for opsonization.

The observation in our investigation that sera from animals with experimental burn wound infection had a greater inhibitory effect on bacterial phagocytosis by PMNL and a more marked reduction in alternative pathway-mediated complement consumption than non-experimentally infected animals with similar burn sizes suggests that burn wound infection is a major contributing factor in the induction of these abnormalities. This concept is also supported by the finding that these abnormalities were not demonstrated in non-experimentally infected burned animals, until their burn wounds were heavily colonized with bacteria. The extent of burn injury may also play an important role, since the abnormalities were demonstrated in animals with 30% but not 15% total body surface burns. Quantitative burn wound culture results revealed that both groups of animals generally had similar total numbers of bacteria per 100 mg of burn wound tissue. However, the animals with larger burns had twice the surface area colonized, and pathogenic species of bacteria were more prevalent. Thus, it is possible that the larger bacterial load on the burn wounds and/or the prevalence of pathogens led to invasive infection with induction of the abnormalities.

The demonstration in our investigation of elevation of total hemolytic complement occurring prior to the time of maximal burn wound colonization suggests that this alteration is initially related to the inflammatory process associated with burn injury. It is probable that burn wound colonization contributes to perpetuation of this alteration. Reduction in total hemolytic complement in experimental burn wound infection is presumed to reflect complement consumption. These conclusions are consistent with previous reports of elevation of total hemolytic complement accompanying wide-spread inflammatory processes (49-53) and complement consumption occurring during systemic infection (54).

It is difficult to compare the results of this investigation on burned animals to previous results obtained in humans, because numerous variables were not able to be controlled in the human studies. In general, it can be stated that the alterations of total hemolytic complement observed in the burned animals closely parallel those observed in humans with the same extent of third-degree burn injury. In humans, elevation of total hemolytic complement has been observed as early as 5 days postburn and has been shown to persist through early convalescence (11-14); consumption of total hemolytic complement during systemic infection has been well documented in burned humans (11-13).

The temporal sequence of the abnormality of the alternative complement pathway in burned animals appears to differ somewhat from that observed in comparable burned humans. This abnormality has been shown to occur in burned humans during the first 10 days postburn and to continue through early convalescence (11-16); yet, in the burned animals, this abnormality was not demonstrated until ten days postburn and disappeared at the time of early wound healing. Burned humans with infectious complications have been shown to have the most marked reduction in alternative pathway activity (16), and exacerbation of this abnormality was demonstrated in burned animals during experimental burn wound infection.

Serum-mediated inhibition of phagocytosis by PMNL has been shown to be more prevalent in burned animals than humans. This abnormality has been demonstrated in only three of twelve burned humans, appearing after the eighth day postburn and persisting through 4 to 5 weeks postburn in two of the patients and through 7 weeks postburn in the third (28). Patients whose sera contained inhibitory activity had severe infectious complications, but their clinical condition was for the most part similar to that of patients whose sera lacked inhibitory activity (28). Serum-mediated inhibition of phagocytosis in animals with 30% total body surface burns was demonstrated from 10 through 25 days postburn, and exacerbation was documented during experimental burn wound infection. The discrepancy between the animal and human data may be related to the removal of inhibitory factors in the human sera by dialysis, a concept that will be discussed in more detail in the next section of this report. The lack of demonstration of an opsonic defect in the burned animals upon testing of the sera at a concentration of 10% is in agreement with our findings in comparable burned humans (11-14).

The observation in our investigation that experimental burn wound infection with *S. aureus* and *C. albicans* as well as *P. aeruginosa* was associated with exacerbation of serum-mediated inhibition of phagocytosis and abnormal alternative pathway activity tends to rule out a role for classical endotoxin in their mediation. Consumption of total hemolytic complement was demonstrated at the time of exacerbation of the abnormalities, yet the abnormalities were observed in non-experimentally infected animals in association with elevation of total hemolytic complement. This latter observation and the failure to demonstrate significant correlations between the abnormalities and total hemolytic complement in non-experimentally infected animals suggest that the abnormalities are not mediated by cleavage products of complement components. Our hypothesis regarding mediators of the abnormalities will be discussed in more detail in the next section of this report.

B. Human Studies

1. Results

a. Relationship between nutritional status and serum-mediated inhibition of PMNL bactericidal activity and reduction in alternative pathway-mediated C3 conversion

During the project period, 11 burned patients were enrolled in our study. The group included ten males and one female ranging in age from 17

to 49 years. All patients had flame burn injuries, and the total burn sizes ranged from 20% to 75%. All patients were admitted to the Cincinnati General Hospital on the day of burn injury. Pertinent clinical characteristics of the patients are presented in Table 3.

Clotted blood specimens were collected as soon as informed consent was obtained. In seven of the patients, sampling was initiated during the first week postburn; informed consent was not given on four of the patients until the second or third week postburn, at which time sampling was started. One or two blood specimens were obtained at weekly intervals providing the patient's condition was stable. Sampling was continued until the patients were fully grafted, discharged, or died. Blood specimens were allowed to clot for 1 hour at room temperature and were centrifuged at $5,000 \times g$ for 15 minutes at 4°C . The sera were removed, divided into small aliquots, and frozen at -70°C .

The ability of the sera to inhibit the bactericidal activity of normal human PMNL was measured by a minor modification of our published method (28). Equal parts of *S. aureus* 502A or *E. coli* 075 (1.0×10^9 cfu/ml) and pooled normal human serum or GHBSS were incubated for 10 minutes at 37°C . The bacteria were washed once and resuspended in GHBSS to a final concentration of 1.0×10^8 cfu/ml. Test sera were dialyzed for 18 hours at 4°C against 0.01M phosphate buffered saline, pH 7.4, containing 0.15 mM CaCl_2 and 0.5 mM MgCl_2 . Reaction mixtures consisted of bacteria opsonized with pooled normal human serum (2.0×10^6 cfu/ml), normal human PMNL (1.0×10^7 cells/ml), and 98% of the test serum. Controls consisted of opsonized or GHBSS-treated bacteria and PMNL. The reaction mixtures and controls were rotated at 37°C , and total surviving bacteria were enumerated at 0 time and after 30 and 60 minutes of incubation. The percent of bactericidal activity was determined by the formula $\frac{a-b}{a} \times 100$, where a was equal to the total number of surviving bacteria at 0 time and b was equal to the total number of surviving bacteria after 30 or 60 minutes of incubation. The percent of inhibition of PMNL bactericidal activity was calculated by the same formula, where a was equal to the percent of PMNL bactericidal activity in the presence of pooled normal human serum and b was equal to the percent of PMNL bactericidal activity in the presence of the test serum. Conversion of C3 by inulin and CoVF in the sera was measured as described previously (16).

Sufficient data were available to estimate the nutritional status of eight of the newly enrolled patients and two previously studied patients whose sera were shown to inhibit the bactericidal activity of normal human PMNL. The clinical characteristics of these latter two patients were presented in reference 28; they were designated Patients 2 and 11 in this previous report. For the purposes of this report, these patients will be referred to as I and II. Enteral and parenteral routes were utilized to administer nutrients to all patients. Nursing personnel encouraged the intake of high caloric oral supplements at frequent intervals, and all patients also received liquid high-protein, high-caloric solutions via an indwelling nasogastric feeding tube. Standard tables were used to estimate caloric values of protein and non-protein nutrients in enteral foods, and standard conversion formulas were used to estimate caloric values of carbohydrate and protein in parenteral solutions. The estimated daily caloric

Table 3. Clinical Characteristics of the Patients.

Patient No.	Age	Sex ^a	Body Surface Injured(%) ^b		Pulmonary Injury ^c	Postburn Day ^d	Infectious Complications		
			Total	Third Degree			Type	Microorganism	Outcome
1	35	M	20	0	N	13	Catheter-related sepsis	<u>C. albicans^e</u>	Lived
2	33	M	67	9	N	6	Pneumonia	<u>S. pneumoniae</u>	Lived
3	43	M	55	10	M	8	Catheter-related sepsis	<u>E. cloacae^e</u>	Lived
4	28	M	59	11	M	6	Pneumonia	<u>S. aureus</u> <u>E. cloacae</u>	Lived
						34	Pneumonia	<u>P. aeruginosa</u>	
5	49	M	58	12	M	5	Catheter-related sepsis	<u>S. aureus^e</u>	Lived
						20	Catheter-related sepsis	<u>C. albicans^e</u>	
						50	Pneumonia	<u>K. pneumoniae</u> <u>P. mirabilis</u> <u>S. marcescens</u>	
						53	Bacteremia	<u>S. aureus^e</u>	
						57	Intraabdominal abscess	<u>B. fragilis^e</u> <u>E. coli^e</u> <u>K. pneumoniae</u>	
6	25	M	56	13	N	4	Catheter-related sepsis	<u>S. aureus</u>	Lived

Table 3. Clinical Characteristics of the Patients (cont'd).

Patient No.	Age	Sex ^a	Body Surface Injured(%) ^b		Pulmonary Injury ^c	Postburn Day ^d	Infectious Complications		
			Total	Third Degree			Type	Microorganism	Outcome
7	18	M	54	14	M	13	Burn wound sepsis	<u>S. aureus</u> ^e	Lived
						21	Catheter-related sepsis	<u>P. aeruginosa</u>	
8	47	M	50	15	N	5	Catheter-related sepsis	<u>S. aureus</u> ^e	Lived
						46	Bacteremia	<u>S. aureus</u> ^e	
9	20	F	42	35	M	10	Catheter-related sepsis	<u>S. aureus</u>	Died (day 41, sepsis)
						16	Burn wound sepsis	<u>P. aeruginosa</u> ^e	
						30	Pneumonia	<u>P. aeruginosa</u>	
10	17	M	56	56	M	13	Burn wound sepsis	<u>S. aureus</u> ^e <u>P. aeruginosa</u>	Lived
						47	Viremia	Cytomegalovirus	
11	26	M	75	60	S	5	Burn wound sepsis	<u>S. aureus</u> ^e	Died (day 11, respiratory insufficiency and sepsis)
						7	Pneumonia	<u>E. coli</u>	

^a M = male; F = female.

^b All patients had flame burn injuries.

^c Degree of pulmonary injury as documented by bronchoscopy (S = severe; M = moderate; N = none).

^d Postburn day complication was apparent.

^e Microorganism was recovered from blood cultures.

requirement for each patient was calculated using the formula described by Cuerreri et al. (55). The daily requirement of protein was determined from the estimated daily caloric requirement assuming protein was required on the basis of 150 Kcal/g N (56). The results were expressed as percent of estimated total caloric or protein requirement and were calculated by dividing weekly intake by weekly requirement and multiplying by 100.

Sera from only one of the newly enrolled patients (No. 11) inhibited the bactericidal activity of PMNL. Inhibitory activity was demonstrated in the serum sample obtained during the first week postburn; inhibition was 47% after 30 minutes of incubation and 21% after 60 minutes of incubation. Sera obtained during the second week postburn from this patient did not contain detectable inhibitory activity. Total caloric and protein intake in this patient during the time when inhibitory activity was demonstrated were not lower than values obtained during the same time period from several of the other patients whose sera lacked inhibitory activity (Table 4). The two previously studied patients whose sera contained inhibitory activity during 3 through 8 weeks postburn (Patient I) and 2 through 5 weeks postburn (Patient II) were as nutritionally replete during this time as the majority of patients whose sera lacked inhibitory activity.

The relationships between total caloric or protein intake and C3 conversion by inulin and CoVF were also investigated. C3 conversion values for each week were averaged, when data had been derived from multiple serum samples. These values and the weekly values of total caloric and protein intake were subjected to arcsin transformation (46) prior to computing correlation coefficients. Data obtained during weeks 1 through 4 and weeks 5 through 8 were analyzed separately. The data are presented in Figures 22-25. r^2 did not exceed 0.29 for any of the computations, suggesting that the correlations were weak. Similar analyses revealed insignificant correlations between immunochemical concentrations of C3 and total caloric or protein intake.

It was next of interest to determine the relationship between cumulative total caloric or protein deficits and reduction in C3 conversion by inulin and CoVF. Patient I was excluded from these computations, since caloric and protein intake was not calculated during the first 2 weeks postburn. Total caloric and protein intake based on estimated requirements were calculated from the day of admission to the time of collection of each serum sample. These values and the individual values of C3 conversion were subjected to arcsin transformation, and correlation coefficients were computed. r^2 was less than 0.14 for all computations. These results and those described in the preceding paragraph suggested that reduction in C3 conversion was not related to poor nutritional status.

b. Effects of heat treatment, ammonium sulfate fractionation, and incubation with benzamidine hydrochloride on burn serum inhibitor(s) of PMNL bactericidal activity

Two patients were included in this aspect of our investigation, on whom sufficient sera were available to perform the determinations. The first was Patient II described in the preceding section, and the second was designated Patient III for the purposes of this report. Clinical characteristics of this latter patient (previously referred to as Patient 12) have been presented in our published work (28). Prior to each experiment,

Table 4. Total Caloric and Protein Intake in the Patients during the Study Period^a.

Patient No. ^c	Total Caloric Intake (%) / Protein Intake (%) ^b							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	27/14	84/77	45/46	100/88				
2	35/23	44/26	42/27	78/70	95/95			
3	31/41	57/63	55/71	86/66	81/66	100/76		
7	35/25	64/60	100/97	52/38	47/34	56/39	48/42	100/96
8	54/43	78/74	100/86					
9	22/29	38/26	38/49	62/57	46/79	25/37		
10	26/17	79/61	83/73	90/63	95/88	72/56	67/37	95/95
11	28/36	17/30						
I			39/13	70/26	45/10	86/26	69/37	54/35
II	28/34	72/73	90/82	93/71	74/84	56/47	67/67	61/74

^a Intake was calculated for the duration of the study or until intake approximated the estimated requirement and remained stable.

^b Values were calculated by dividing weekly intake by weekly estimated requirement and multiplying by 100.

^c Patients I and II were designated 2 and 11 in our previously published study (28).

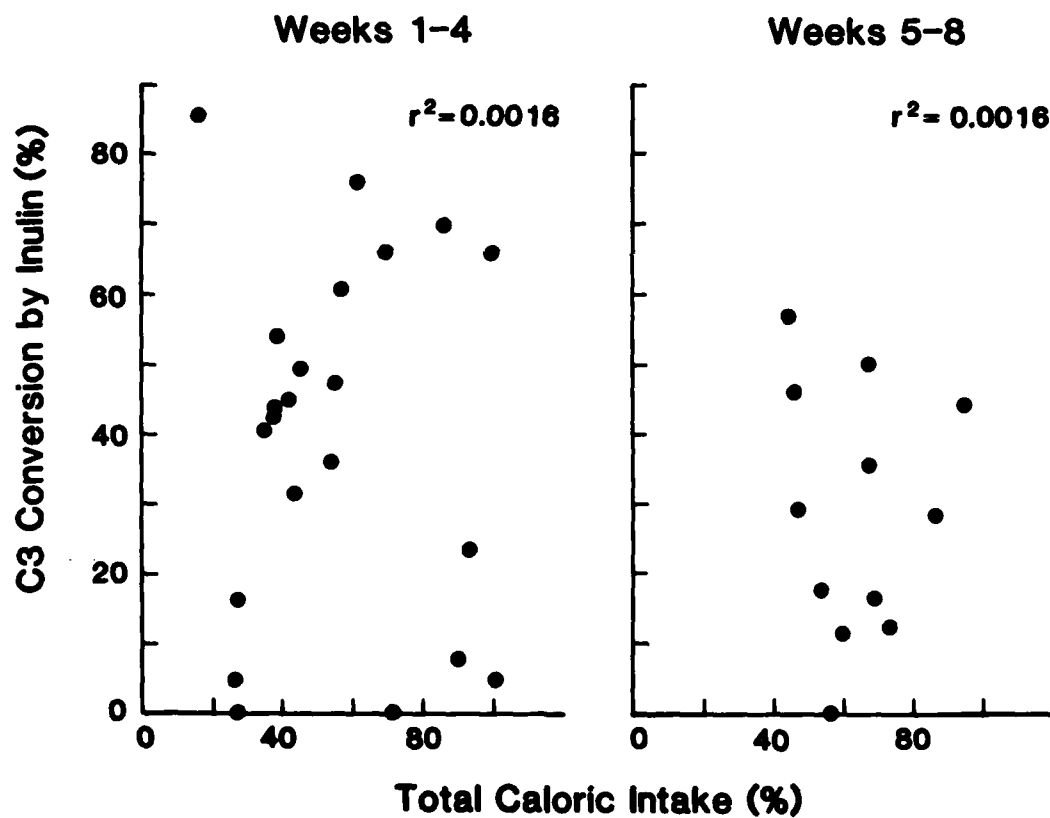


Figure 22. Relationship between C3 conversion by inulin and total caloric intake during 1 through 4 and 5 through 8 weeks postburn.

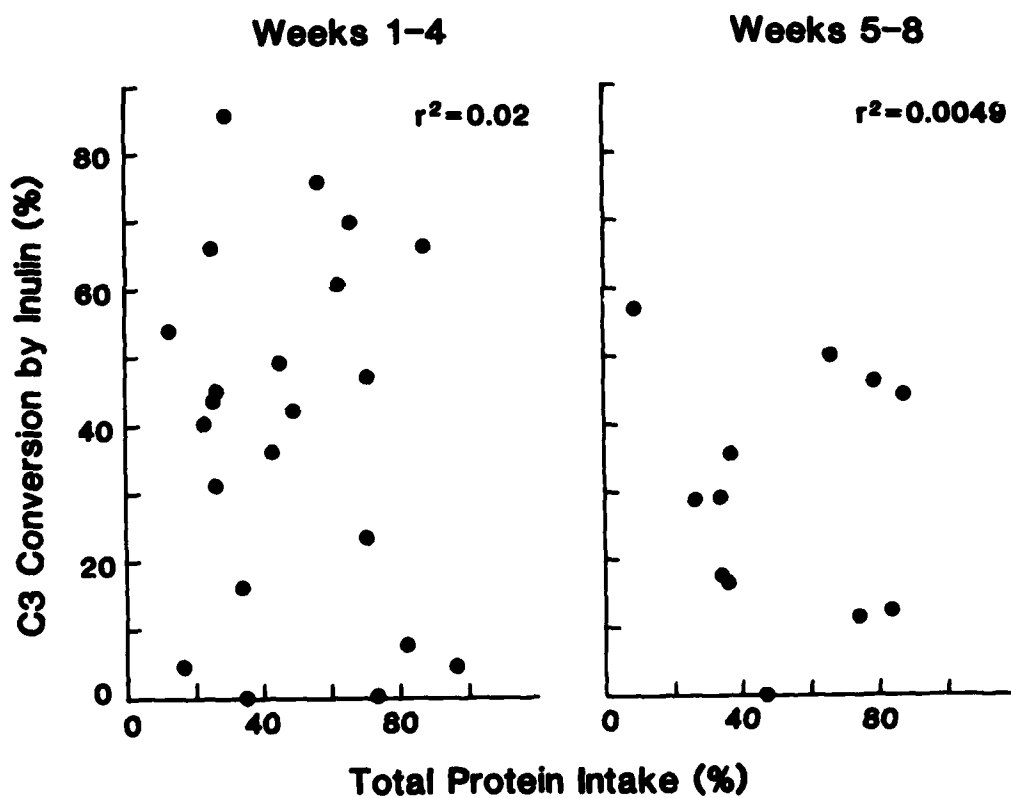


Figure 23. Relationship between C3 conversion by inulin and protein intake during 1 through 4 and 5 through 8 weeks postburn.

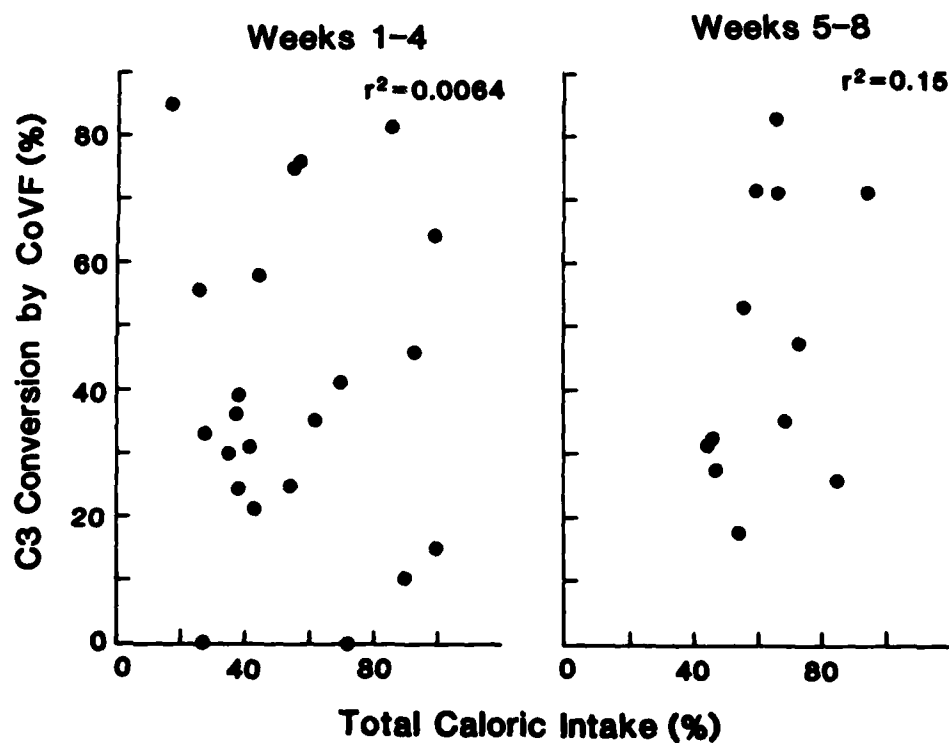


Figure 24. Relationship between C3 conversion by CoVF and total caloric intake during 1 through 4 and 5 through 8 weeks postburn.

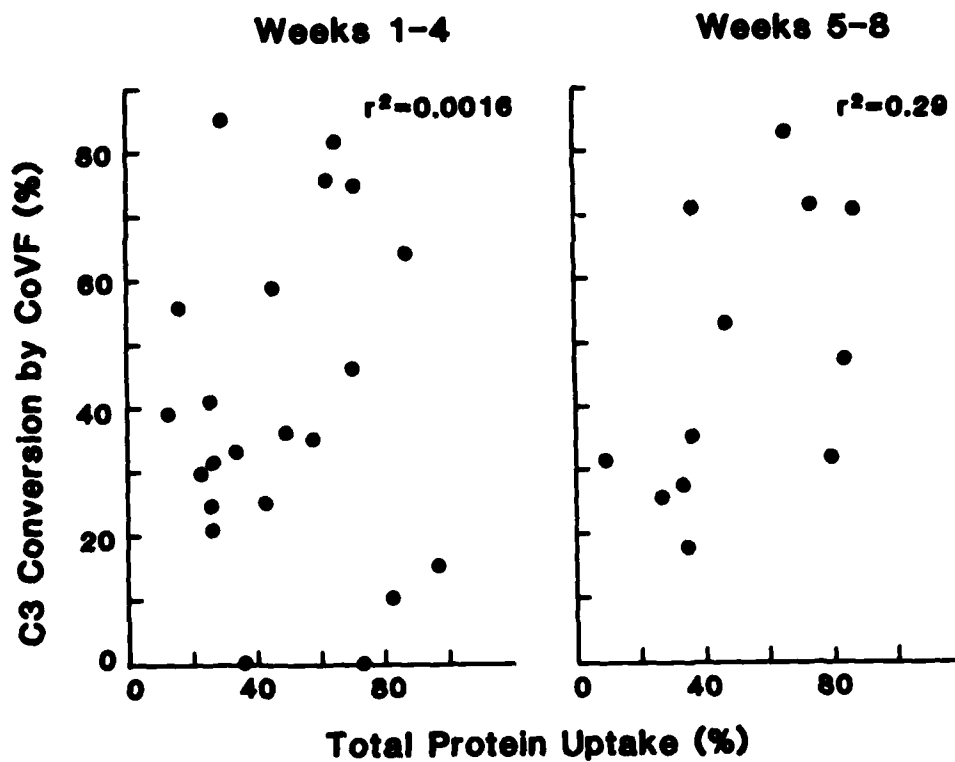


Figure 25. Relationship between C3 conversion by CoVF and protein intake during 1 through 4 and 5 through 8 weeks postburn.

equal parts of sera obtained from Patient II during 16 through 30 days postburn and from Patient III during 13 through 50 days postburn were pooled separately. Each patient's pooled serum was dialyzed for 18 hours at 4°C against 0.01 M phosphate buffered saline, pH 7.4, containing 0.15 mM CaCl_2 and 0.5 mM MgCl_2 . The methodology for measurement of PMNL bactericidal activity and the formula for calculating percent of inhibition described in the preceding section were used in all experiments; S. aureus 502A was used as the bacterial test strain. In preliminary experiments, both patients' sera were found to contain inhibitory activity.

Initial experiments were performed to determine the heat stability of the inhibitory activity. Patients' sera and pooled normal human serum were heated for 30 minutes at 56°C, and PMNL bactericidal activity in the presence of heated and untreated sera was compared. Inhibition of PMNL bactericidal activity by heated sera was equivalent to that observed with untreated sera (Table 5). These results suggested that the inhibitory activity in the burn sera was heat-stable.

The demonstration that the inhibitory activity was heat-stable lead us to postulate that it might be mediated by IgG antibodies directed against glycoproteins of the PMNL membrane. To test this hypothesis, the burn sera and pooled normal human serum were subjected to 50% ammonium sulfate fractionation, and the fractions were tested for their inhibitory effect on PMNL bactericidal activity. A volumetric modification of the method of Chase was used to fractionate the sera with ammonium sulfate (57). The test sera (500 μl) were mixed with 500 μl of ice-cold 0.15 M NaCl, pH 7.1. Saturated ammonium sulfate (1 ml) was added, and the solution was stirred gently for 2 hours at 4°C. The precipitates were centrifuged, washed once with 2 ml of 40% ammonium sulfate, and resuspended in 500 μl of distilled water. The solubilized samples were then dialyzed extensively at 4°C against distilled water and then GHBSS. Negligible inhibition of PMNL bactericidal activity was demonstrated with the ammonium sulfate fractions as compared with inhibition observed with unfractionated burn sera (Table 6). These negative results suggested that the inhibitory activity was distinct from IgG.

The ability of benzamidine hydrochloride to reverse the inhibitory effect of the burn sera on PMNL bactericidal activity was also investigated. Serum from Patient II and pooled normal human serum (392 μl) were incubated for 30 minutes at 37°C with 3.2 μl of distilled water or 50 mM benzamidine hydrochloride prepared in distilled water. The sera were then dialyzed for 18 hours at 4°C against isotonic veronal buffered saline (VBS), pH 7.4, containing 0.15 mM CaCl_2 , 0.5 mM MgCl_2 , and 0.1% gelatin (GVB⁺⁺). The inhibitory effect of the sera on PMNL bactericidal activity was compared with that of untreated sera. Inhibition at 30 minutes of incubation in the presence of the benzamidine hydrochloride-treated burn serum was greater than that observed with the control-treated or untreated burn serum (Table 7). At 60 minutes of incubation, the benzamidine hydrochloride-treated burn serum inhibited PMNL bactericidal activity to the same extent as the control-treated burn serum. These results suggested that the inhibitory effect of the burn serum was not related to the presence of proteases sensitive to inactivation by benzamidine hydrochloride with PMNL receptor-cleaving potential.

Table 5. Effect of Heat Treatment on the Inhibitory Effect of Burn Sera on the Bactericidal Activity of Normal Human PMNL^a.

<u>Patient Designation</u>	<u>Treatment of Serum</u>	<u>Inhibition (%)</u>	
		<u>30 min.</u>	<u>60 min.</u>
II	None	59	60
	$\Delta 56^{\circ}\text{C}$, 30 min	50	49
III	None	50	32
	$\Delta 56^{\circ}\text{C}$, 30 min	45	32
Pooled normal human serum	$\Delta 56^{\circ}\text{C}$, 30 min.	0	0

^a Results of a representative experiment are presented.

Table 6. Effect of Ammonium Sulfate Fractionation on the Inhibitory Effect of Burn Sera on the Bactericidal Activity of Normal Human PMNL^a.

<u>Patient Designation</u>	<u>Treatment of Serum</u>	<u>Inhibition (%)</u>	
		<u>30 min.</u>	<u>60 min.</u>
II	Unfractionated	47	53
	50%(NH ₄) ₂ SO ₄	4	0
III	Unfractionated	39	22
	50%(NH ₄) ₂ SO ₄	5	0
Pooled normal human serum	50%(NH ₄) ₂ SO ₄	0	0

^a Results of a representative experiment are presented.

Table 7. Effect of Incubation with Benzamidine Hydrochloride on the Inhibitory Effect of Burn Serum on the Bactericidal Activity of Normal Human PMNL^a.

<u>Source of Serum</u>	<u>Treatment of Serum</u>	<u>Inhibition (%)^b</u>	
		<u>30 min.</u>	<u>60 min.</u>
Patient II	None	43	43
	distilled H ₂ O	33	29
	benzamidine HCl	62	21
Pooled normal human serum	distilled H ₂ O	0	0
	benzamidine HCl	0	0

^a Results of a single experiment are presented.

c. Investigation of the mechanism responsible for reduction in alternative pathway-mediated C3 conversion

Previous studies conducted in our laboratory failed to demonstrate a correlation between reduction in alternative pathway-mediated C3 conversion in burn sera and elevation of immunochemical concentrations of H or I, or both proteins (Annual Summary Report, June, 1978). This observation suggested that reduction in C3 conversion was not caused by elevation of these regulatory proteins of the alternative pathway. However, we hypothesized that the burn sera might contain a factor capable of augmenting inactivation of surface-bound C3b by H and I and thus inhibiting alternative pathway-mediated C3 conversion. Experiments were therefore designed to test this hypothesis.

The sera that were used for these experiments were obtained from five previously studied burned patients, which were designated A-E for the purposes of this report. The ages, sex, and burn sizes of the patients are presented in Table 8. Equal parts of sera obtained from the patients during 8 weeks postburn were pooled separately, and C3 conversion by inulin and CoVF was measured in each patient's pooled serum as described in Section IV.B.1.a. C3 conversion by both activating substances in the burn sera was reduced as compared with C3 conversion in pooled normal human serum (Table 9).

The ability of dilutions of the burn sera and pooled normal human sera to inactivate erythrocyte-bound C3b and thus prevent convertase formation with isolated alternative pathway proteins was determined by a modification of the method of Medicus et al. (58). C3 (59), B (60), D (61), and P (62) were isolated from human serum in functionally purified form. Sheep erythrocytes (5.0×10^8 cells) and 150 μ g of C3 in a final volume of 550 μ l of GVB⁺⁺ were prewarmed to 37°C. Trypsin (3.7 μ g) was added, and the cell suspension was incubated for 3 minutes at 37°C. The cells designated E-C3b (try) were washed once and resuspended in VBS containing 0.01 M EDTA and 0.1% gelatin (0.01 M GVBE). E-C3b (try) (5.0×10^6 cells) in a volume of 30 μ l were incubated for 10 minutes at 37°C with 30 μ l of two-fold dilutions (1:10-1:80) of test serum in 0.01 M GVBE. 0.01 M GVBE was substituted for test serum for preparation of control cells. The cells were washed once and resuspended in GVB⁺⁺. The treated erythrocytes (5.0×10^6 cells) were incubated for 3 minutes at 37°C with B (5 μ g), D (0.05 μ g), P (0.5 μ g), and GVB⁺⁺ in a final volume of 120 μ l. The reaction was stopped by the addition of 40 μ l of 0.04 M GVBE. Rat serum diluted 1:15 in 0.04 M GVBE (160 μ l) was added as a source of C3-C9, and the reaction was continued for 60 minutes at 37°C. The tubes were centrifuged, and hemoglobin in the supernatants was measured spectrophotometrically at 415nm. Controls included a reagent blank in which GVB⁺⁺ was substituted for B, D, and P and a water lysate (320 μ l) of 5.0×10^6 E-C3b (try). The absorbance of the reagent blank was subtracted from the absorbance of the test samples. The percent of lysis was calculated. The number of hemolytic sites per erythrocyte (Z) was calculated by the formula $-\ln(1-y)$, where y was equal to the percent of lysis. The results were then expressed as the dilution of test serum which yielded a Z value of 1.0.

Table 8. Ages, Sex, and Burn Sizes of the Previously Studied Patients whose Sera were Tested in the Experiments Described in Section IV.B.1.c.

<u>Patient Designation</u>	<u>Age</u>	<u>Sex^a</u>	<u>Body Surface Injured (%)^b</u>	
			<u>Total</u>	<u>Third Degree</u>
A	57	M	45	1
B	38	F	28	10
C	49	M	40	10
D	46	F	23	22
E	21	M	51	47

^a M = male; F = female.

^b All patients had flame burn injuries.

Table 9. C3 Conversion by Inulin and CoVF in Pooled Sera from Patients A-E.

<u>Patient Designation</u>	<u>C3 Conversion (%)</u>	
	<u>Inulin</u>	<u>CoVF</u>
A	51	81
B	36	52
C	32	38
D	32	58
E	35	44
Pooled normal human serum	68	91

Immunochemical concentrations of H and I in the sera were measured by single radial immunodiffusion (63). Agarose (1%) dissolved in veronal buffer ($\mu = 0.05$, pH = 8.6) containing 0.04 M EDTA was used. Goat anti-serum to human H was kindly provided by Dr. Clark D. West, Children's Hospital Research Foundation, Cincinnati, OH. Goat antiserum to human I was prepared as described previously (11).

Higher dilutions of four of the five burn sera facilitated inactivation of erythrocyte-bound C3b as compared with pooled normal human serum (Table 10). However, these dilutions as expressed in percent of normal were not higher than the average of the immunochemical concentrations of H and I in the sera. Thus, all of the inactivation observed could be attributed to the concentrations of H and I. This observation ruled out the possibility that a factor capable of augmenting the functions of H and I might be present in the burn sera causing reduction in C3 conversion.

Modulation of alternative pathway activation is dependent on the opposing actions of the regulatory proteins, H and I, and the proteins required for convertase formation, C3, B, and D (64,65). Inhibition of alternative pathway activation has been demonstrated upon addition of modest concentrations of H and I to normal human serum, and the inhibition has been overcome by supplementation of the serum with C3 and B (64). The physiological concentration of D is also limiting; lowering and raising the concentration has been shown to result in diminution and augmentation of alternative pathway activation respectively (65). In light of these observations, we theorized that aberrations in the relative concentrations of the five alternative pathway proteins might be responsible for the reduction in alternative pathway-mediated C3 conversion in burn sera.

Two serum samples from Patients 1, 2, 4, and 9 were used in our experiments. The first sample was obtained during the first 16 days postburn, and the second sample was obtained during 19 through 23 days postburn. Immunochemical concentrations of H and I in the sera were measured as described above. Quantitation of immunochemical concentrations of C3 and B was also performed by single radial immunodiffusion using antisera and conditions described previously (11). Functional activity of D was quantitated by a minor modification of the method of Lesavre et al. (66). D-depleted serum was prepared by passage of 30 ml of fresh human serum containing 0.01 M EDTA over a column (2 x 20 cm) of Bio-Rex 70 equilibrated with VBS; the breakthrough was collected and dialyzed extensively against VBS. Fifteen μ l of D-depleted serum, 6 μ l of a solution containing 0.1 M EGTA and 0.1 M $MgCl_2$, 10 μ l of rabbit erythrocytes (1.0×10^7 cells), 80 μ l of VBS containing 0.1% gelatin (GVB), and 10 μ l of two-fold dilutions of test serum (1:2-1:16) in GVB were incubated for 12 minutes at 37°C. The reaction was stopped by the addition of 1 ml of ice-cold GVB. The tubes were centrifuged, and hemoglobin in the supernatants was measured spectrophotometrically at 415nm. Controls included a reagent blank in which GVB was substituted for the test serum and a water lysate (1.121 ml) of 1.0×10^7 rabbit erythrocytes. The absorbance of the reagent blank was subtracted from the absorbance of the test samples, and the percent of lysis was determined. Z values were calculated, and the results were expressed as the dilution of test serum which yielded a Z value of 1.0. Final results were expressed as percent of normal and were calculated by dividing the dilution of test serum at 12 by the dilution of pooled nor-

Table 10. Comparison of Functional Activity and Average Immunochemical Concentration of H and I in Burn Sera with Reduced C3 Conversion^a.

<u>Patient Designation</u>	<u>Inactivation of Erythrocyte-Bound C3b</u>		<u>Immunochemical</u>
	<u>Serum Dilution at 1 Z</u>	<u>% of Normal</u>	$\frac{H + I}{2}$ <u>(% of Normal)</u>
A	67	156	161
B	51	119	119
C	57	133	151
D	64	149	156
E	40	93	132
Pooled normal human serum	43	100	100

^a Results of a representative experiment are presented.

mal human serum at 1:2 and multiplying by 100. In preliminary experiments, supplementation of D-depleted serum with a physiological concentration of highly purified D (66) isolated in our laboratory facilitated complete lysis of the rabbit erythrocytes.

Reduction in C3 conversion in the burn sera was generally associated with supranormal immunochemical concentrations of C3, B, H, and I (Table 11). The ratios of H and I to C3 and B fell within the normal range, suggesting that reduction in C3 conversion was not related to elevation of H and I as compared with C3 and B. A significant correlation between C3 conversion by both activating substances and functional D activity was demonstrated (Figure 26). These preliminary results suggested that reduction in C3 conversion in the burn sera was associated with diminution in the functional activity of D.

d. Effects of burn sera on mitogen-induced lymphocyte transformation

Experiments were performed to determine if burn sera with reduced C3 conversion inhibited mitogen-induced transformation of normal human PBL. Sera from Patients A-E described in the preceding section were used in these experiments.

A minor modification of the method of Artz et al. (67) was used to measure lymphocyte transformation. PBL were obtained from heparinized blood of healthy adult donors, separated on Ficoll-Hypaque, and washed three times with Hanks's balanced salt solution. The cells were resuspended in RPMI 1640 containing 5% heat-inactivated (56°C, 30 minutes) fetal calf serum and 0.1% gentamicin (culture medium), and viability was determined by trypan blue dye exclusion. Mixtures of cells and test serum were prepared by supplementing 2 ml of cells (2.0×10^6 cells/ml) with 145 or 290 μ l of test serum. Culture medium was substituted for test serum in the controls. Two-hundred μ l samples of the cell suspensions were dispensed in triplicate into flat-bottomed Microtest culture plates. Purified Phytohemagglutinin (PHA) and Concanavalin A (Con A) were added in 50 μ l volumes at final concentrations of 0.05, 0.1, 0.5, and 1.0 μ g per culture well (PHA) and 0.1, 0.2, and 2.0 μ g per culture well (Con A). Triplicate cell samples were also cultured in the absence of mitogen. The plates were incubated for 72 hours at 37°C in a humidified atmosphere of 5% CO₂. Two hours prior to cell harvest, 0.2 μ Ci of ³H-thymidine (specific activity = 6.7 Ci/mmol) in a volume of 50 μ l were added to each culture. Cells were collected on glass fiber filter strips by an automatic Mash II sample harvester, and uptake of radioactivity was measured in a Beckman LS 7000 liquid scintillation counter. Initial results were expressed as mean cpm \pm the standard error of the mean. Mean values were used when the data on individual test sera were grouped, and the standard error of the mean was derived from these values. Selected data were expressed as percent inhibition which was calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to mean cpm in the cultures lacking serum and b was equal to mean cpm in the cultures containing test serum.

In initial experiments, proliferative responses of normal PBL to increasing concentrations of PHA in the presence of 5% or 10% of burn or normal sera were compared. The normal sera used in these experiments were collected from five healthy adult donors and were handled identically to

Table 11. Immunochemical Concentrations of C3, B, H, and I in Burn Sera with Reduced C3 Conversion.

Patient Designation	Days Postburn ^a	C3 Conversion (%)		Immunochemical Concentration (% of Normal)				$\frac{H + I}{C3 + B}$ ^b
		Inulin	CoVF	C3	B	H	I	
1	16	52	74	152	94	110	106	0.88
	23	66	64	164	192	170	170	0.96
2	7	40	30	153	170	158	208	1.13
	19	45	31	158	156	105	170	0.88
4	6	41	37	122	138	100	125	0.87
	19	40	37	138	128	126	170	1.11
9	9	44	25	141	138	110	170	1.00
	21	34	31	114	144	105	118	0.86

^a Time of collection of serum sample.

^b Ratios in five healthy adult donors ranged from 0.8 to 1.38 (mean = 1.06).

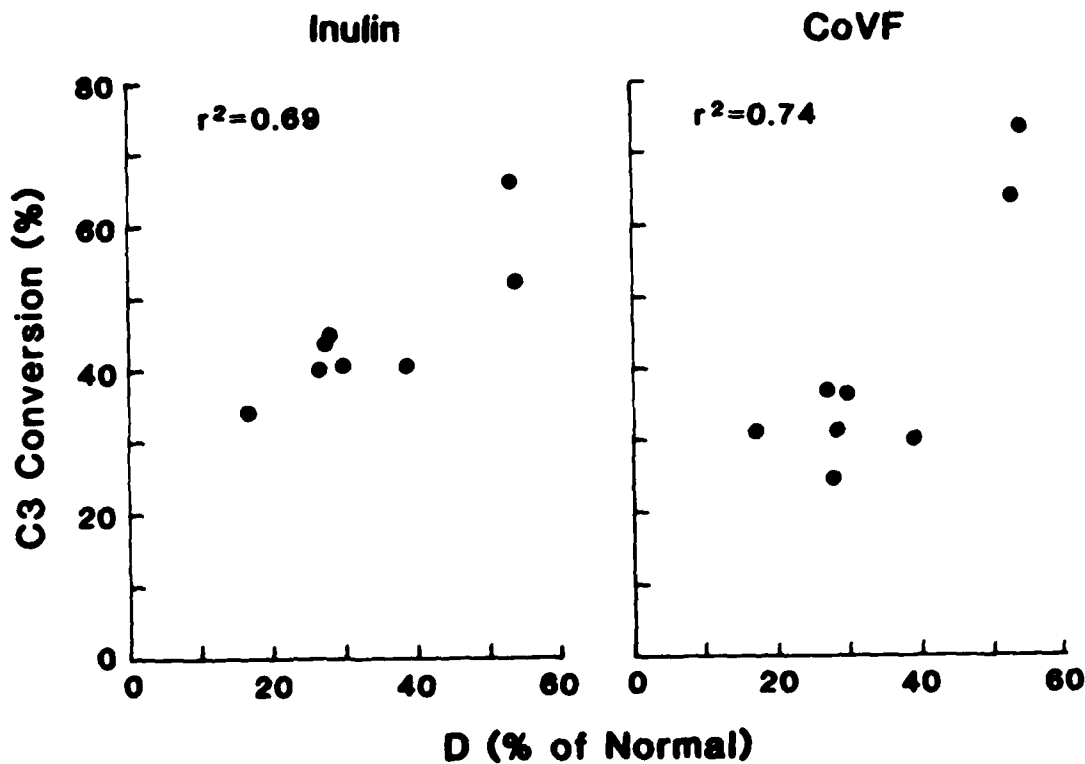


Figure 26. Relationship between C3 conversion by inulin and CoVF and functional activity of D in the burn sera. P values for both determinations were less than 0.005. Range of D activity in sera from five healthy adult donors was 77% to 114% (mean = 93%).

the burn sera. Proliferative responses in the presence of the sera were equivalent at all doses of mitogen, and these responses paralleled those observed in the absence of serum (Figure 27). Differences in proliferative responses in the presence of 10% of the sera were also not observed using a different leukocyte donor, although the responses were lower than those observed in the first series of experiments (Figure 28). Proliferative responses in the presence of the sera were slightly higher than those observed in the absence of serum. Testing of doses of mitogen less than 0.05 μ g per culture well yielded greater variation of results, and substantive differences in proliferative responses in the presence of burn and normal sera were not demonstrated.

The effect of the sera on Con A-induced transformation of normal PBL was next investigated. At all doses of mitogen proliferative responses in the presence of 10% of the burn sera were reduced as compared with those observed in the presence of the same concentration of normal sera or in the absence of serum (Figure 29). Subsequent results showed that sera from Patients B and E were the least inhibitory at all doses of mitogen (Table 12). Maximal inhibitory activity in all sera was demonstrated at the highest doses of mitogen (1.0 or 2.0 μ g per culture well).

Pooled serum from Patient II which inhibited the bactericidal activity of normal human PMNL was also tested for its inhibitory effect on lymphocyte transformation by PHA and Con A; preparation of the serum has been described in Section IV.B.1.b. Proliferative responses in the presence of the patient's serum were equivalent to those observed with pooled normal human serum at doses of PHA ranging from 0.05 μ g to 1.0 μ g per culture well. Inhibition of PBL proliferation at doses of 0.2 μ g and 2.0 μ g of Con A per culture well was 44% and 55% respectively.

e. Fractionation of burn serum inhibitor(s) by DEAE-cellulose chromatography

Sera from Patients A and C and pooled normal human serum were chromatographed on DEAE-cellulose using a minor modification of the method by Nimberg et al. (68). The patients' sera were identical to those used in the experiments described in preceding sections of this report. Serum (2 ml) was adjusted to pH 5.2 with 7% glacial acetic acid and then applied to a 1.5 x 17 cm column of DEAE-cellulose equilibrated with 0.005 M sodium acetate buffer, pH 5.0. The column was washed with equilibrating buffer, until the absorbance at 280 nm was negligible. Protein was eluted by step-wise addition of 0.03 M, 0.1 M, and 0.2 M sodium acetate buffer, pH 5.0. The flow rate was 10 ml per hour, and 5 ml fractions were collected. A representative elution profile is shown in Figure 30. The four protein peaks were concentrated by lyophilization, solubilized in distilled water, and subjected to diafiltration using Amicon UM-05 membranes and five volumes of 0.01 M phosphate buffered saline, pH 7.0. The peaks were millipore filtered and stored in small aliquots at -70°C. Protein was quantitated by the method of Lowry et al. (69).

Initial experiments were performed to determine the inhibitory effect of the peaks on lymphocyte transformation by Con A. Suspensions of normal PBL in culture medium (2.0×10^6 cells/ml) were supplemented with 5%-20% (v/v) of peak, 0.01 M phosphate buffered saline, pH 7.0, or culture

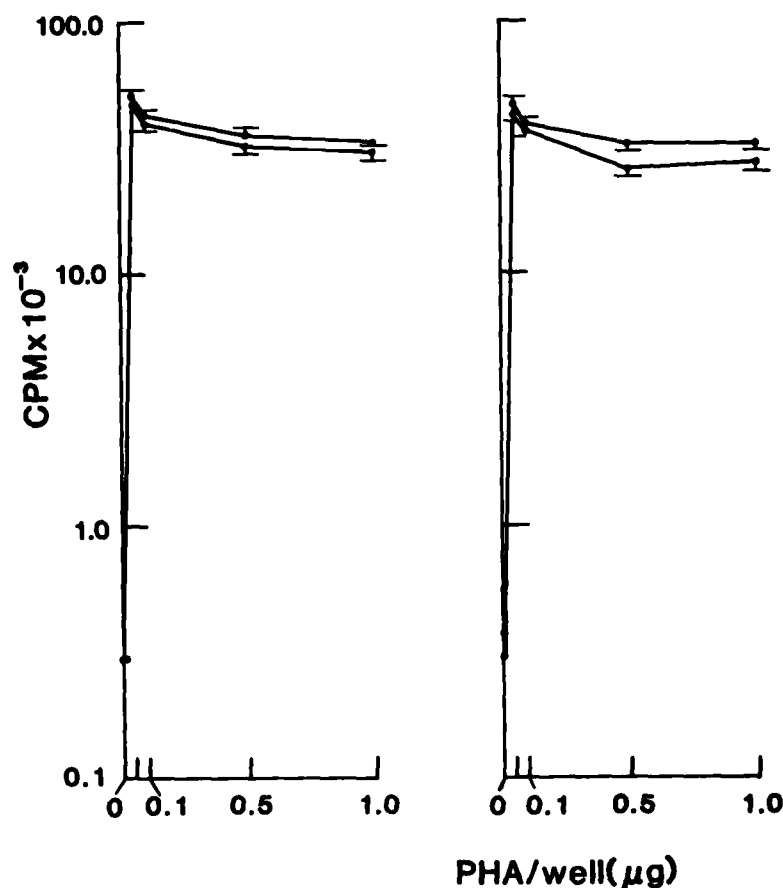


Figure 27. Proliferative responses of normal human PBL to increasing concentrations of PHA in the presence of 5% (left figure) or 10% (right figure) of burn (●) or normal (○) sera. The points represent mean values of determinations performed with five sera, and each vertical bar represents the standard error of the mean. Responses in the absence of serum in the two experiments were as follows: no mitogen (246 ± 40 , 573 ± 115), $0.05 \mu\text{g}$ ($44,424 \pm 1620$, $43,660 \pm 2,586$), $0.1 \mu\text{g}$ ($36,257 \pm 1,396$, $40,594 \pm 9,379$), $0.5 \mu\text{g}$ ($30,703 \pm 484$, $23,821 \pm 1,636$), and $1.0 \mu\text{g}$ ($21,798 \pm 1,344$, $28,263 \pm 6,669$).

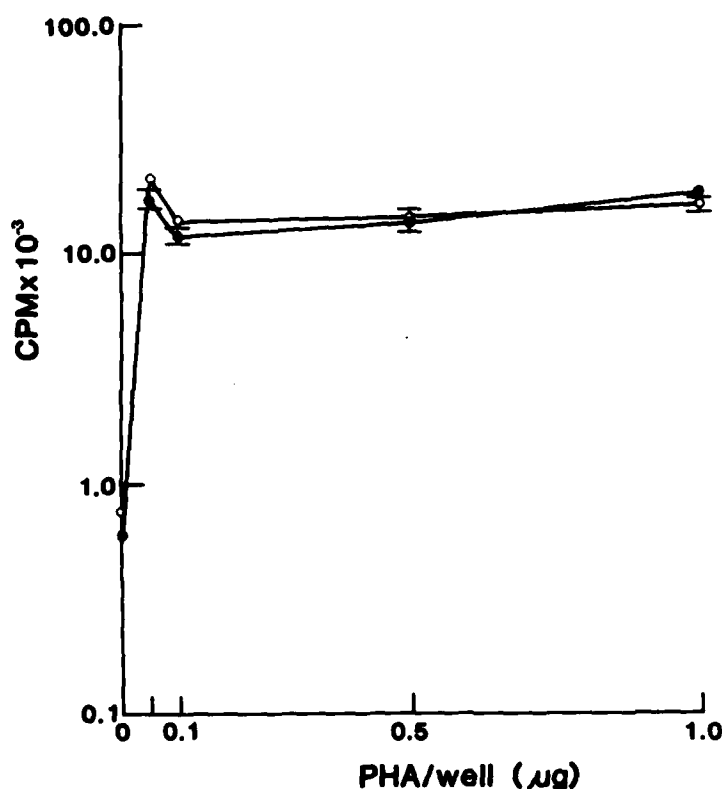


Figure 28. Proliferative responses of normal human PBL to increasing concentrations of PHA in the presence of 10% of burn (●) or normal (○) sera. PBL were harvested from a different donor than was used in the experiments presented in Figure 27. The points represent mean values of determinations performed with five sera, and each vertical bar represents the standard error of the mean. Responses in the absence of serum were as follows: no mitogen (493 ± 41), $0.05 \mu\text{g}$ ($15,793 \pm 510$), $0.1 \mu\text{g}$ ($9,379 \pm 326$), $0.5 \mu\text{g}$ ($7,185 \pm 240$), and $1.0 \mu\text{g}$ ($7,336 \pm 583$).

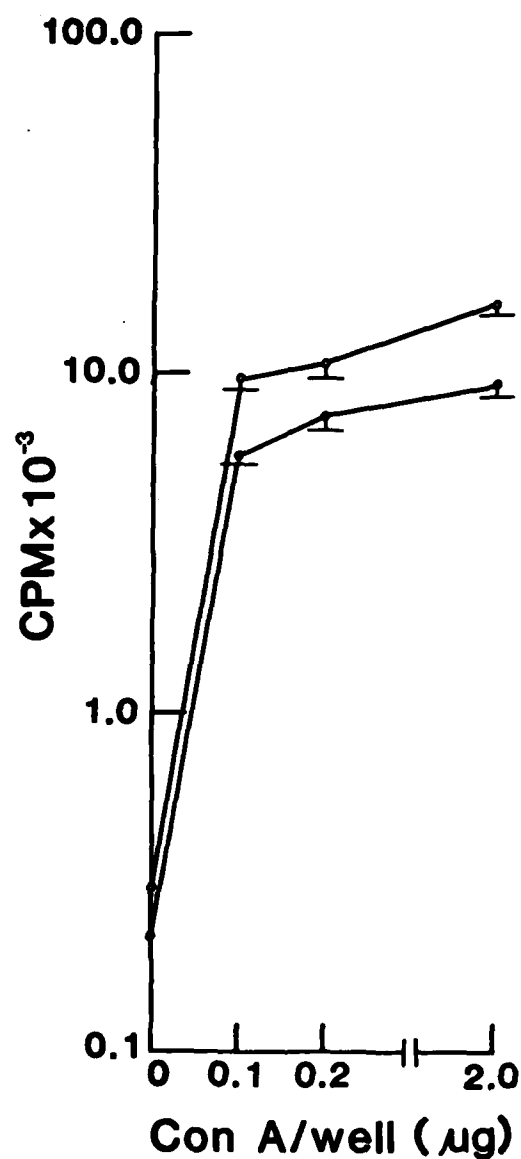


Figure 29. Proliferative responses of normal human PBL to increasing concentrations of Con A in the presence of 10% of burn (●) or normal (○) sera. The points represent mean values of determinations performed with four normal sera and five burn sera, and each vertical bar represents the standard error of the mean. Responses in the absence of serum were as follows: no mitogen (326 ± 99), 0.1 µg ($10,282 \pm 388$), 0.2 µg ($10,702 \pm 162$), and 2 µg ($18,277 \pm 817$).

Table 12. Inhibitory Effect of the Burn Sera on Con A-Induced Transformation of Normal Human PBL^a.

<u>Patient Designation</u>	<u>Inhibition (%) ^b</u>			
	<u>Con A/well(μg)</u>			
	<u>0.2</u>	<u>0.5</u>	<u>1.0</u>	<u>2.0</u>
A	24	40	54	41
B	0	5	4	35
C	14	39	22	53
D	27	46	40	46
E	3	16	18	35

^a Sera were tested at a concentration of 10% per well.

^b Percent inhibition was calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to mean cpm in the cultures without serum and b was equal to mean cpm in the cultures containing test serum. Responses in the absence of serum were as follows: no mitogen (534 ± 87), 0.2 μg (11,976 ± 416), 0.5 μg (14,754 ± 368), 1.0 μg (17,620 ± 593), and 2.0 μg (26,375 ± 3,881).

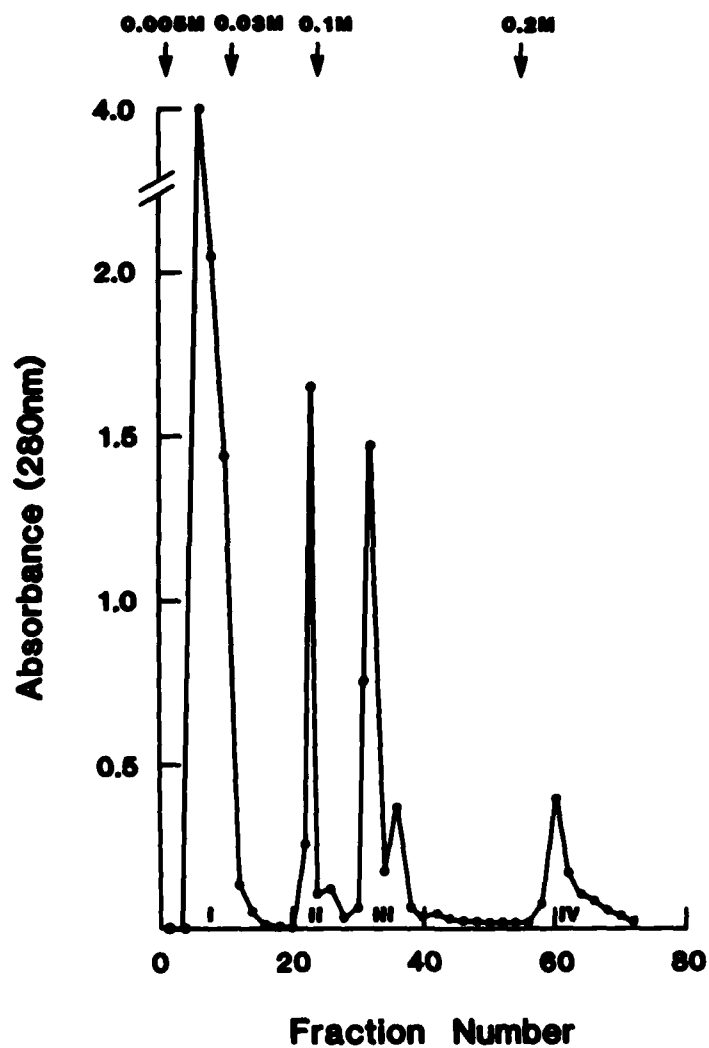


Figure 30. Elution profile after chromatography of human serum on DEAE-cellulose. Serum from Patient A was used in this experiment.

medium. Culture conditions were as described previously, and Con A was used at a concentration of 0.2 μ g per culture well. The percent of inhibition was calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to mean cpm in the cultures containing culture medium and b was equal to mean cpm in the cultures containing test peak. In two experiments, additional wells were prepared in duplicate containing 200 μ l of cell suspension and 50 μ l of mitogen. These cultures were used for assessment of cell viability. Viability was determined by counting 200 cells using fluorescein diacetate-ethidium bromide staining (70).

In preliminary experiments, proliferative responses to Con A in the presence and absence of phosphate buffered saline were found to be equivalent. Maximal inhibition of the proliferative response was demonstrated with Peak IV fractionated from patients' or control sera (Table 13). The inhibitory effect was observed upon supplementation of the cell suspensions with 30 μ g of protein per ml. Inhibition was greater with the peaks fractionated from the patients' sera as compared with the peak fractionated from the control serum. Peak III from Patient B's serum also inhibited the proliferative response when tested at this protein concentration. Inhibition was negligible with Peak III fractionated from Patient A's serum or the control serum and Peaks I and II fractionated from all sera. Testing of Peaks I, II, and III at a concentration of 100 μ g of protein per ml of cell suspension revealed augmented inhibition with Peak III fractionated from Patient B's serum and substantive inhibition with Peak I isolated from this serum. Negligible inhibition was observed with the other peaks. A four-fold or greater decrease in cpm in cultures containing Peak IV but no mitogen was observed. In addition, decrease in viability was associated with inhibition of the proliferative response to Con A by Peak IV. Decrease in viability was greater in the second experiment as compared with the first experiment. These results taken together suggest that the inhibitory effect of Peak IV on lymphocyte transformation was attributed in part to cell death.

The inhibitory effect of the peaks on C3 conversion by CoVF in pooled normal human serum was next investigated. Pooled normal human serum (89 μ l) was supplemented with 11 μ l of test peak to yield a final concentration of 30 μ g of protein per ml. Two 40 μ l aliquots of supplemented serum were removed to separate tubes. Four μ l of CoVF (100 units/ml) were added to one of the aliquots; the other aliquot served as the control for detection of peak-mediated complement consumption and was not further supplemented. The concentration of B antigen of C3 was quantitated in the aliquots at 0 time and after 60 minutes of incubation at 37°C (16). Initial results were expressed in percent of C3 conversion and were calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to the concentration of B antigen at 0 time and b was equal to the concentration of B antigen after 60 minutes of incubation. Selected data were expressed as percent of inhibition of C3 conversion and were calculated by the same formula, where a was equal to the percent of C3 conversion by CoVF in unsupplemented serum and b was equal to the percent of C3 conversion by CoVF in serum supplemented with test peak.

Table 13. Inhibitory Effect of Peaks I - IV on the Proliferative Response of Normal Human PBL to Con A.

<u>Serum Fractionated</u>	<u>Peak No.</u>	<u>Inhibition (%)^a</u>			<u>Viability(%)</u>	
		<u>Exp. 1^b</u>	<u>Exp. 2^b</u>	<u>Exp. 3^c</u>	<u>Exp. 1^b</u>	<u>Exp. 2^b</u>
Patient A	I	0	1	13	86	79
	II	0	0	5	89	80
	III	6	10	2	89	86
	IV	99	98	-	77	42
Patient B	I	0	10	55	82	80
	II	0	2	2	85	77
	III	63	59	97	91	79
	IV	99	99	-	81	35
Pooled normal human serum	I	0	8	14	86	77
	II	0	0	0	86	84
	III	0	0	0	80	87
	IV	89	81	-	76	60

^a Responses in the presence of culture medium and mitogen were $13,571 \pm 1,862$ (Exp. 1), $7,900 \pm 282$ (Exp. 2), and $9,600 \pm 532$ (Exp. 3); viability was 92% in Exp. 1 and 2.

^b Peak I-IV were tested at a final concentration of 30 $\mu\text{g/ml}$ of cell suspension.

^c Peaks I, II, and III were tested at a final concentration of 100 $\mu\text{g/ml}$ of cell suspension. Viability was not determined.

None of the peaks facilitated C3 consumption in pooled normal human serum in the absence of CoVF. Peak IV fractionated from the patients' sera inhibited C3 conversion by CoVF in pooled normal human serum (Table 14). Inhibition was negligible with Peak IV fractionated from pooled normal human serum. Inhibition was also not detected with Peaks I, II, and III isolated from patients' or control sera.

The bactericidal activity of normal human PMNL was also measured in the presence of the peaks. Reaction mixtures consisted of *S. aureus* 502A opsonized with pooled normal human serum (2.0×10^6 cfu/ml), normal human PMNL (1.0×10^7 cells/ml), and 98% of unsupplemented GHBSS or GHBSS supplemented with test peak (30 μ g of protein/ml). The reaction mixtures were rotated at 37°C, and total surviving bacteria were enumerated at 0 time and after 30 and 60 minutes of incubation. The percent of bactericidal activity was determined after 30 and 60 minutes of incubation as described in Section IV.B.1.a. The percent of inhibition of PMNL bactericidal activity was calculated by the formula $\frac{a-b}{a} \times 100$, where a was equal to the percent

of PMNL bactericidal activity in the presence of unsupplemented GHBSS and b was equal to the percent of PMNL bactericidal activity in the presence of GHBSS supplemented with test peak.

Peak IV fractionated from the patients' sera inhibited PMNL bactericidal activity at 30 and 60 minutes of incubation (Table 15). Peak IV fractionated from pooled normal human serum inhibited PMNL bactericidal activity at 60 minutes but not at 30 minutes of incubation; the inhibitory effect at 60 minutes of incubation was less than that observed with Peak IV isolated from the patients' sera. Negligible inhibition of PMNL bactericidal activity was demonstrated with Peak I, II, and III isolated from patients' or control sera.

Testing of PMNL bactericidal activity in the presence of unfractionated serum from Patients A and C using the conditions described in Section IV.B.1.a. failed to detect an inhibitory effect. Thus, the demonstration of inhibition by Peak IV fractionated from these sera was unexpected. However, unfractionated sera had been dialyzed to remove antibiotics prior to testing using 12,000 molecular weight retention tubing, whereas dialyzable factors of similar size had not been removed from the peaks. Thus, it was possible that the dialysis procedure had removed factors of low molecular weight with an inhibitory effect on PMNL bactericidal activity. Because the burn sera no longer were available in sufficient quantities to use in additional experiments, we compared the ability of increasing concentrations of dialyzed and non-dialyzed pooled normal human serum to inhibit the bactericidal activity of normal PMNL. We theorized that the dialyzable factors were present in pooled normal human serum in a concentration lower than that in burn serum thereby explaining the ability of Peak IV isolated from non-dialyzed pooled normal human serum to inhibit minimally PMNL bactericidal activity.

PMNL bactericidal activity was measured as described above in the presence of 80%, 90%, and 98% of dialyzed or non-dialyzed pooled normal human serum. The percent of inhibition of PMNL bactericidal activity was calculated by the formula described above, where a was equal to PMNL bactericidal activity in the presence of 98% of GHBSS and b was equal to PMNL

Table 14. Inhibitory Effect of Peaks I - IV on C3 Conversion by CoVF in Pooled Normal Human Serum^a.

<u>Serum Fractionated</u>	<u>Peak No.</u>	<u>Inhibition (%)</u>
Patient A	I	0
	II	0
	III	0
	IV	68
Patient B	I	0
	II	0
	III	0
	IV	49
Pooled normal human serum	I	0
	II	0
	III	0
	IV	3

^a Results of a representative experiment are presented.

Table 15. Inhibitory Effect of Peaks I - IV on the Bactericidal Activity of Normal Human PMNL^a.

<u>Serum Fractionated</u>	<u>Peak No.</u>	<u>Inhibition (%)</u>	
		<u>30 min.</u>	<u>60 min.</u>
Patient A	I	0	6
	II	0	19
	III	0	0
	IV	53	54
Patient B	I	0	0
	II	8	6
	III	0	0
	IV	79	75
Pooled normal human serum	I	0	1
	II	0	4
	III	0	19
	IV	0	41

^a Results of a representative experiment are presented.

bactericidal activity in the presence of the various concentrations of dialyzed or non-dialyzed pooled normal human serum. Inhibition at 30 and 60 minutes of incubation was demonstrated at all concentrations of non-dialyzed serum and ranged from 10% to 36%. Inhibition was maximal at the highest concentration of serum (36% at 30 minutes and 34% at 60 minutes). An inhibitory effect of dialyzed serum was not demonstrated at any of the serum concentrations tested. These results provided support for the concept that naturally occurring dialyzable factors inhibit the bactericidal activity of PMNL.

2. Discussion

From the results of our investigation, the following conclusions can be drawn: a) Reduction in alternative complement pathway-mediated C3 conversion and serum-mediated inhibition of PMNL bactericidal activity in burned patients are not related to poor nutritional status. b) Burn serum inhibitor(s) of PMNL bactericidal activity are heat-stable and distinct from IgG and proteases with sensitivity to inactivation by benzamidine hydrochloride. c) Reduction in alternative pathway-mediated C3 conversion in burn sera is not caused by an aberrant factor that facilitates inactivation of surface-bound C3b by H and I or by an elevation of the concentrations of H and I as compared with C3 and B. Rather, this abnormality is associated with diminution in the functional activity of D. d) Reduction in functional D activity may be related to the presence of alpha globulin-associated factor(s) that also inhibit PMNL bactericidal activity and lymphocyte transformation by Con A.

The immunosuppressive activity of naturally occurring alpha globulin-associated factors is well documented. Alpha globulin isolated from human or animal sera has been shown to prolong allograft survival (71-75), inhibit lymphocyte transformation in vitro by mitogens, antigens, and allogeneic cells (75-79), suppress primary and secondary antibody responses in vivo and in vitro (74, 79-88), inhibit rosette formation (84, 85, 88, 89), enhance tumor growth (90-93), and inhibit antigen-mediated macrophage immobilization (94), proliferation of human cell lines of B or T origin (95), and various cytotoxic reactions (74, 96, 97) in vitro. Evidence has been presented to suggest that alpha globulin affects early events in antigen recognition by lymphocytes (77) without causing cell death as assessed by trypan blue dye exclusion (76-79, 83, 85).

Inhibition by human alpha globulin of mouse peritoneal leukocyte and whole blood bactericidal activity associated with increased susceptibility to experimental streptococcal infection has also been demonstrated (74, 85, 88). The inhibitory effect of alpha globulin on phagocytic cell function was reversed by washing of the leukocytes (85). Higher concentrations of alpha globulin were found to be required to suppress phagocytic function as compared with lymphocyte function (74, 88).

Elevation of alpha globulin has been documented in mice after antigenic stimulation (86, 87) and in humans undergoing renal graft rejection (98). Elevated alpha globulin in ataxia telangiectasia has been associated with reduced lymphocyte proliferative responses to PHA and pokeweed mitogen (99). Immunosuppressive activity in cancer sera has been related to altered alpha-2 macroglobulin in several studies (100, 101) and

to an alpha globulin-associated peptide in other studies (68, 102). In these latter studies, the peptide was isolated primary in the breakthrough after chromatography of serum on DEAE-cellulose using the conditions described in our investigation. The peptide was shown to be chemically similar to that associated with the alpha globulin fraction of normal human serum (79). It was postulated that carrier sites on the alpha globulin became saturated with the peptide, which then became associated with non-alpha globulin proteins. Chromatographically similar material with immunosuppressive activity has been isolated from the sera of burned patients (35, 103) and patients following major operative and non-burn accidental trauma (104, 105).

In our investigation, the inhibitory effect of burn sera on lymphocyte transformation by Con A was mediated primarily by the alpha globulin fraction. At equal protein concentration, the alpha globulin fraction of the burn sera inhibited lymphocyte transformation to a greater extent than the alpha globulin fraction of normal human serum. Inhibitory activity in one of the two burn sera tested was also isolated in the breakthrough as described previously (35, 103). It is possible that this material carries the immunosuppressive peptide normally associated with the alpha globulin fraction or contains presumably distinct immunosuppressive factors such as micromolecular fibrinogen degradation products (106) or C3a (107, 108). However, it is doubtful that the active factor is C3a, since C3a has not been shown to inhibit lymphocyte proliferative responses in fetal calf serum-containing culture medium (109, 110).

The lack of demonstration in our investigation of an inhibitory effect of the burn sera on lymphocyte transformation by PHA remains unexplained. Suboptimal and optimal doses of PHA were tested, yet inhibition of the responses by burn sera was not observed. Reinherz et al. have demonstrated that lymphocytes bearing T4 or T5 antigens respond equally well to Con A, whereas lymphocytes bearing T5 respond poorly to PHA (111). An inducer or helper role has been assigned to the T4⁺ subset in T-T, T-B, and T-macrophage interactions (112). In contrast, the T5⁺ subset contains cells with major cytotoxic and suppressor functions (112). It is attractive to speculate that burn sera may preferentially affect the T5⁺ subset resulting in augmented suppressor activity. This hypothesis would also explain the greater inhibitory effect of burn sera on lymphocyte proliferative responses to Con A as compared with PHA.

Decrease in viability was associated with alpha globulin-mediated inhibition of lymphocyte transformation in our investigation but not in those reported previously. This finding may be related to the difference in sensitivity of fluorescein diacetate-ethidium bromide staining and trypan blue dye exclusion in detecting cell death. We suspect that concentrations of alpha globulin lower than those used in our experiments inhibit lymphocyte transformation without altering cell viability, although this hypothesis has not yet been tested experimentally. It is also possible that alpha globulin exerts its effect by selectively killing a subpopulation of cells required for maximal proliferative responses.

The concentrations of alpha globulin required to inhibit phagocytic cell function appear to be higher than those required to inhibit lymphocyte function as suggested originally by Glaser et al. (74,88). In our investigation, a concentration of normal human alpha globulin that markedly

inhibited lymphocyte transformation by Con A failed to inhibit phagocytic cell function. This difference may be related to a greater sensitivity of lymphocytes to alpha globulin or to the use of twenty-five times more cells in the phagocytic assay as compared with the lymphocyte culture assay. The observation that alpha globulin fractionated from burn sera inhibited phagocytic cell function is presumed to be related to elevation of inhibitory alpha globulin-associated factors. Our preliminary data suggests that these factors may be dialyzable and more prevalent in human burn sera than previously realized. This concept is supported by our data in animals which were derived from testing of non-dialyzed sera (refer to section IV.A.1.b).

In a previous report from our laboratory, it was shown that burn serum-mediated inhibition of PMNL bactericidal activity was related to an inhibitory effect of the burn sera on the phagocytic process which reduced the number of bacteria internalized and killed intracellularly (16). Preliminary evidence was also presented to suggest that the inhibitory effect of the burn sera was not associated with cell death and was not reversed by washing of the leukocytes. This latter observation appears to conflict with the concept that the inhibitory activity may be associated with alpha globulin, since alpha globulin-associated inhibition of phagocytic cell function has been shown to be readily reversed by washing (84). Further research is needed to clarify this discrepancy, to prove that alpha globulin-associated factors in burn sera are responsible for inhibition of PMNL, lymphocyte, and complement function, and to establish the mechanisms by which these factors exert their effect.

V. CONCLUSIONS

Burn wound infection is a contributing factor in the induction of serum-mediated inhibition of PMNL function and reduction in alternative complement pathway activity following burn injury. Elevation of total hemolytic complement is initially related to the inflammatory process associated with burn injury, and it is probable that burn wound colonization contributes to perpetuation of this alteration.

Serum-mediated inhibition of PMNL function and reduction in alternative complement pathway activity following burn injury are not related to poor nutritional status.

Burn serum inhibitor(s) of PMNL function are heat-stable and distinct from IgG and proteases with sensitivity to inactivation by benzamidine hydrochloride.

Reduction in alternative pathway activity in burn sera is not caused by an aberrant factor that facilitates inactivation of surface-bound C3b by H and I or to elevation of the concentrations of H and I as compared with C3 and B. Rather, this abnormality is associated with diminution in the functional activity of D.

Burn serum inhibitor(s) of PMNL, lymphocyte, and alternative complement pathway function are alpha globulin-associated factors.

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